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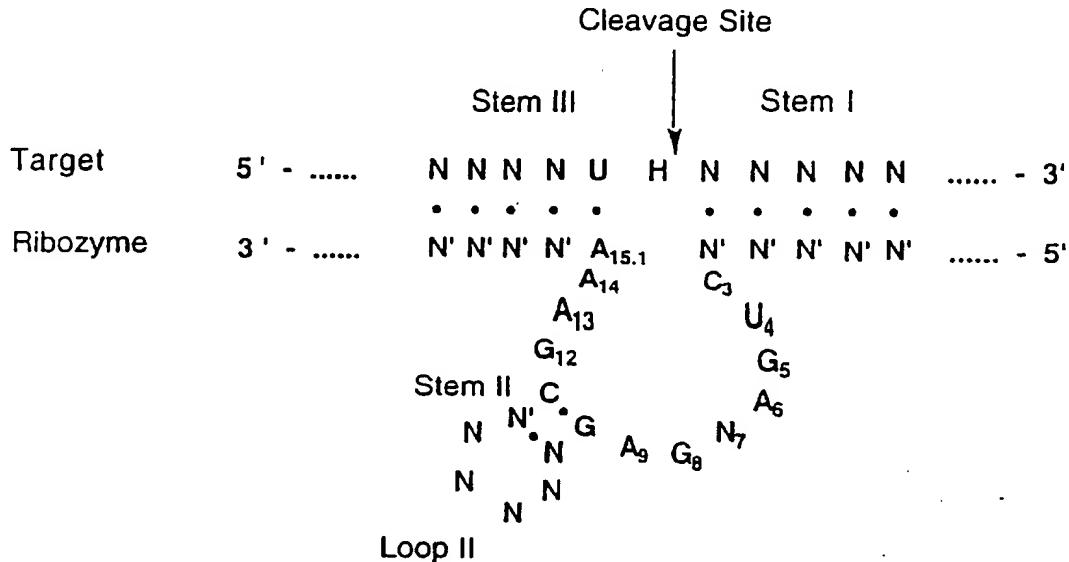
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(54) Title: ENZYMATIC NUCLEIC ACID TREATMENT OF DISEASES OR CONDITIONS RELATED TO LEVELS OF C-FOS



(57) Abstract

Enzymatic nucleic acid molecules which cleave c-fos RNA.

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DESCRIPTIONEnzymatic Nucleic Acid Treatment Of Diseases Or  
Conditions Related To Levels Of C-FOSBackground Of The Invention

5       The present invention concerns therapeutic compositions and methods for the treatment of cancer.

The present invention relates to therapeutic compositions and methods for the treatment or diagnosis of diseases or conditions related to c-fos expression levels, such as cancer. The discussion is not meant to be complete and is provided only for understanding of the invention that follows. This summary is not an admission that any of the work described below is prior art to the claimed invention.

15      The c-fos proto-oncogene encodes a transcription factor that plays an important regulatory role in the response to mitogenic stimuli (for a review see Angel et al., 1991, *Biochem. Biophys. Acta* 1072, 129). Evidence in the literature indicates that c-fos is necessary for expression of many matrix metallo-proteinases (MMPs), including stromelysin 1, stromelysin 2, collagenase 1, matrylisin, 92 kD gelatinase and human macrophage metalloelastase (Sato et al., 1993, *Oncogene* 8, 395; Gaire et al. 1994 *J Biol Chem* 269, 2032; Lauricelle-  
25 Lefebvre et al. 1993 *Invasion Metastasis* 13, 289; Belaaouaj et al. 1995 *J Biol Chem* 270, 14568). C-fos also regulates the expression of other proteases including urokinase-type plasminogen activator, granzyme B and several cathepsins (Lengyel et al., 1995 *Biochem Biophys Acta* 1268, 65; Troen et al., 1991 *Cell Growth Differ* 2, 23; Hadman et al., 1996 *Oncogene* 12, 135;

Rochefort et al., 1995, *Ciba Found Symp* **191**, 254; Wargnier et al. 1995 *Proc Natl Acad Sci, USA* **92**, 6930). Applicant believes that the implications of several of these proteases in tumor metastasis indicates that  
5 inhibition of c-fos has the potential to reduce invasive phenotype, as claimed herein. In addition to regulating protease expression, c-fos is necessary for expression of tissue factors, which play an important role in angiogenesis (Felts et al., 1995, *Biochemistry* **34**, 12355; Contrino et al., 1996, *Nature Med* **2**, 209). C-fos  
10 is required for expression of the mdr-1 gene (multi-drug resistance), which is thought to contribute to failures in chemotherapy (Scanlon et al., 1994, *Proc Natl Acad Sci, USA* **91**, 11123). C-fos has been shown to play a  
15 role in cell proliferation in some cell types (Rijnders et al., 1985, *Biochem Biophys Res Comm* **132**, 548). There is also some suggestion that c-fos may have a role in neuronal injury, degeneration, cell death and/or neoplasms (Schlingensiepen et al., International PCT  
20 Publication No. WO 95/02051).

The proto-oncogene c-fos is the cellular homolog of the v-fos gene from FBJ murine osteosarcoma virus. Members of the Fos protein family (c-fos, fosB, fra-1 and fra-2) form heterodimers with members of the jun  
25 family (c-jun/AP-1, junB and junD). The heterodimers act as transcriptional activators by binding DNA at AP-1 sites present in a variety of genes, including collagenase, IL-2, adipocyte P2, human metallothionein IIA, transin, and the DNA repair enzymes thymidylate  
30 (dTMP) synthase, DNA polymerase  $\beta$ , and topoisomerase I. Expression of c-fos is normally tightly regulated at both the RNA and protein level. The kinetics of expression follow the classic pattern of an immediate

early gene; mRNA levels peak at 30-45 minutes following mitogenic stimulation and thereafter decline rapidly. The *c-fos* gene contains an AT-rich mRNA destabilizing sequence in 3' non-coding region, giving the mRNA a 5 half-life of about 12 minutes. The Fos protein has a relatively short half-life (under 2 hours) and negatively regulates transcription of the *c-fos* gene, contributing to rapid down-regulation (Morgan et al., 1991 *Annu Rev Neurosci.* **14**, 421-451; Ransone et al., 10 1990, *Annu Rev Cell Biol.* **6**, 539).

The connection between *fos* expression and osteosarcoma was first suggested by the identification of *v-fos* in murine osteosarcoma virus. Greater than 90% of mice infected with the *fos* viruses FBJ-MSV and FBR-15 MSV develop bone tumors. It appears that deregulated expression of the normal *c-fos* gene can result in similar oncogenic transformation. For example, over-expression of *c-fos* in tissue culture cells yields a transformed phenotype, and in transgenic mice results in 20 a high frequency of bone and cartilage tumors. The majority of human osteosarcomas (HOS) exhibit significantly elevated *c-fos* levels (Wu et al. 1990 *Oncogene* **5**, 989). Unlike *ras*, no specific *c-fos* mutations have been identified that correlate with 25 oncogenic potential.

Transgenic mice that constitutively express *c-fos* develop normally until a few weeks after birth, when bone hyperplasia becomes evident (Ruther et al., 1989 *Oncogene* **4**, 861). Approximately 20% develop bone 30 tumors. The level of *c-fos* expression is at least 10-fold higher in tumor tissue compared to normal tissue. Interestingly, although constitutive expression of *c-fos* occurs in many tissues, lesions are confined to bone

tissue. Thus a secondary tissue-specific event is probably required in addition to elevated *c-fos* levels to bring about malignant transformation. *Fos* expression is also associated with cartilage tumor formation when 5 the transgene is expressed during embryogenesis (Wang et al., 1991 *EMBO J* 10, 2437).

Homozygous *c-fos* knock-out mice are normal at birth, then begin to exhibit osteopetrosis at about 11 days. This is characterized by severe ossification of 10 the marrow space, shortened bones, and absence of tooth eruption due to obstruction by abnormal amounts of bone. In addition, although possessing normal motor skills, the transgenic animals show behavioral abnormalities including hyperactivity and severely diminished response 15 to external stimuli. This is consistent with reports showing that *c-fos* plays a pivotal role in the adaptive responses of the nervous system.

Normal bone is constantly being formed and resorbed by the tightly regulated action of osteoblasts and 20 osteoclasts, respectively. This process is controlled in part by parathyroid hormone (PTH) which differentially affects bone mass depending on whether it is present continuously or intermittently. PTH binds to receptors on osteoblasts and rapidly and transiently 25 induces *c-fos* expression. PTH-activated osteoblasts then induce *c-fos* expression in osteoclasts and bone marrow stromal cells. Thus the temporally-regulated expression of *c-fos* may constitute an essential downstream event in the normal response to PTH. Either 30 deletion or constitutive overexpression of *c-fos* in transgenic mice produces abnormal bone morphology, illustrating the requirement for tightly regulated expression of this protein.

Scanlon, International PCT Publication Nos. WO 91/18624 and WO 96/08558; Scanlon et al., 1991, Proc. Natl. Acad. Sci. USA., 88, 10591; and Funato et al., 1992, Advan. Enzyme Regul., 32, 195, report the use of a 5 hammerhead ribozyme to cleave a site within c-fos mRNA.

Scanlon, International PCT Publication No. WO 96/08558, states on page 9-10 that-

"[D]rug resistance in mammalian, including human, cancer cells is reversed or 10 ameliorated by the down-regulation of the expression of the Fos/Jun heterocomplex and of AP-responsive genes downstream from Fos/Jun in the transduction pathway. Reversal of MDR phenotype by ribozyme 15 suppression of c-fos oncogene expression illustrates one practical application of the invention."

#### Summary Of The Invention

This invention relates to ribozymes, or enzymatic 20 nucleic acid molecules, directed to cleave RNA species that are required for cellular growth responses. In particular, applicant describes the selection and function of ribozymes capable of cleaving RNA encoded by the oncogene, *c-fos*. Such ribozymes may be used to 25 inhibit the hyperproliferation of tumor cells in one or more cancers.

In the present invention, ribozymes that cleave *c-fos* RNA are described. Moreover, applicant shows that 30 these ribozymes are able to inhibit gene expression and cell proliferation *in vitro* and *in vivo*, and that the catalytic activity of the ribozymes is required for their inhibitory effect. From those of ordinary skill

in the art, it is clear from the examples described, that other ribozymes that cleave target RNAs required for cell proliferation may be readily designed and are within the invention.

5 By "inhibit" is meant that the activity of *c-fos* or level of RNAs encoded by *c-fos* is reduced below that observed in the absence of the nucleic acid, particularly, inhibition with ribozymes preferably is below that level observed in the presence of an inactive  
10 RNA molecule able to bind to the same site on the mRNA, but unable to cleave that RNA.

By "enzymatic nucleic acid molecule" it is meant a nucleic acid molecule which has complementarity in a substrate binding region to a specified gene target, and  
15 also has an enzymatic activity which is active to specifically cleave RNA in that target. That is, the enzymatic nucleic acid molecule is able to intermolecularly cleave RNA and thereby inactivate a target RNA molecule. This complementarity functions to  
20 allow sufficient hybridization of the enzymatic nucleic acid molecule to the target RNA to allow the cleavage to occur. One hundred percent complementarity is preferred, but complementarity as low as 50-75% may also be useful in this invention.

25 The term enzymatic nucleic acid is used interchangeably with phrases such as ribozymes, catalytic RNA, enzymatic RNA, catalytic DNA, nucleozyme, DNazyme, RNA enzyme, endoribonuclease, minizyme, leadzyme, oligozyme or DNA enzyme, as used in the art.  
30 All of these terminologies describe nucleic acid molecules with enzymatic activity.

By "equivalent" RNA to *c-fos* is meant to include those naturally occurring RNA molecules associated with cancer in various animals, including human, rat and pig.

By "complementarity" is meant a nucleic acid that can form hydrogen bond(s) with another RNA sequence by either traditional Watson-Crick or other non-traditional types (for example, Hoogsteen type) of base-paired interactions.

Seven basic varieties of naturally-occurring enzymatic RNAs are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds in *trans* (and thus can cleave other RNA molecules) under physiological conditions. Table I summarizes some of the characteristics of these ribozymes. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of an enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

The enzymatic nature of a ribozyme is advantageous over other technologies, since the concentration of ribozyme necessary to affect a therapeutic treatment is lower. This advantage reflects the ability of the ribozyme to act enzymatically. Thus, a single ribozyme molecule is able to cleave many molecules of target RNA. In addition, the ribozyme is a highly specific inhibitor, with the specificity of inhibition depending not only on the base-pairing mechanism of binding to the

target RNA, but also on the mechanism of target RNA cleavage. Single mismatches, or base-substitutions, near the site of cleavage can be chosen to completely eliminate catalytic activity of a ribozyme.

5 Nucleic acid molecules having an endonuclease enzymatic activity are able to repeatedly cleave other separate RNA molecules in a nucleotide base sequence-specific manner. Such enzymatic RNA molecules can be targeted to virtually any RNA transcript, and efficient  
10 cleavage achieved in vitro (Zaug et al., 324, *Nature* 429 1986; Uhlenbeck, 1987 *Nature* 328, 596; Kim et al., 84 *Proc. Natl. Acad. Sci. USA* 8788, 1987; Dreyfus, 1988, *Einstein Quart. J. Bio. Med.*, 6, 92; Haseloff and Gerlach, 334 *Nature* 585, 1988; Cech, 260 *JAMA* 3030,  
15 1988; and Jefferies et al., 17 *Nucleic Acids Research* 1371, 1989).

Because of their sequence-specificity, trans-cleaving ribozymes show promise as therapeutic agents for human disease (Usman & McSwiggen, 1995 *Ann. Rep. Med. Chem.* 30, 285-294; Christoffersen and Marr, 1995 *J. Med. Chem.* 38, 2023-2037). Ribozymes can be designed to cleave specific RNA targets within the background of cellular RNA. Such a cleavage event renders the RNA non-functional and abrogates protein  
20 expression from that RNA. In this manner, synthesis of a protein associated with a disease state can be selectively inhibited.

Ribozymes that cleave the specified sites in *c-fos* RNAs represent a novel therapeutic approach to induce  
30 graft tolerance, treat autoimmune diseases, allergies, cancer and other inflammatory conditions. Applicant indicates that ribozymes are able to inhibit the activity of *c-fos* and that the catalytic activity of the

ribozymes is required for their inhibitory effect. Those of ordinary skill in the art will find that it is clear from the examples described that other ribozymes that cleave these sites in *c-fos* RNAs may be readily 5 designed and are within the scope of this invention.

In one of the preferred embodiments of the inventions herein, the enzymatic nucleic acid molecule is formed in a hammerhead or hairpin motif, but may also be formed in the motif of a hepatitis delta virus, group I 10 intron, group II intron or RNaseP RNA (in association with an RNA guide sequence) or *Neurospora* VS RNA. Examples of such hammerhead motifs are described by Dreyfus, *supra*, Rossi et al., 1992, *AIDS Research and Human Retroviruses* 8, 183; of hairpin motifs by Hampel 15 et al., EP0360257, Hampel and Tritz, 1989 *Biochemistry* 28, 4929, Feldstein et al., 1989, *Gene* 82, 53, Haseloff and Gerlach, 1989, *Gene*, 82, 43, and Hampel et al., 1990 *Nucleic Acids Res.* 18, 299; of the hepatitis delta virus motif is described by Perrotta and Been, 1992 20 *Biochemistry* 31, 16; of the RNaseP motif by Guerrier-Takada et al., 1983 *Cell* 35, 849; Forster and Altman, 1990, *Science* 249, 783; Li and Altman, 1996, *Nucleic Acids Res.* 24, 835; *Neurospora* VS RNA ribozyme motif is described by Collins (Saville and Collins, 1990 *Cell* 25 61, 685-696; Saville and Collins, 1991 *Proc. Natl. Acad. Sci. USA* 88, 8826-8830; Collins and Olive, 1993 *Biochemistry* 32, 2795-2799; Guo and Collins, 1995, *EMBO J.* 14, 363); Group II introns are described by Griffin et al., 1995, *Chem. Biol.* 2, 761; Michels and Pyle, 30 1995, *Biochemistry* 34, 2965; Pyle et al., International PCT Publication No. WO 96/22689; and of the Group I intron by Cech et al., U.S. Patent 4,987,071. These specific motifs are not limiting in the invention and

those skilled in the art will recognize that all that is important in an enzymatic nucleic acid molecule (or multiple fragments of such molecules) of this invention is that it has a specific substrate binding site or 5 arm(s) which is complementary to one or more of the target gene RNA regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule (enzymatic portion).

10 By "enzymatic portion" is meant that part of the ribozyme essential for cleavage of an RNA substrate.

By "substrate binding arm" is meant that portion of a ribozyme which is complementary to (i.e., able to base-pair with) a portion of its substrate. Generally, 15 such complementarity is 100%, but can be less if desired. For example, as few as 10 bases out of 14 may be base-paired. Such arms are shown generally in Figures 1-3 as discussed below. That is, these arms contain sequences within a ribozyme which are intended 20 to bring ribozyme and target RNA together through complementary base-pairing interactions; e.g., ribozyme sequences within stems I and III of a standard hammerhead ribozyme make up the substrate-binding domain (see Figure 1).

25 In a preferred embodiment the invention provides a method for producing a class of enzymatic cleaving agents which exhibit a high degree of specificity for the RNA of a desired target. The enzymatic nucleic acid molecule is preferably targeted to a highly conserved 30 sequence region of a target mRNAs encoding c-fos proteins such that specific treatment of a disease or condition can be provided with either one or several enzymatic nucleic acids. Such enzymatic nucleic acid molecules can be delivered exogenously to specific cells

as required. Alternatively, the ribozymes can be expressed from DNA/RNA vectors that are delivered to specific cells.

Synthesis of nucleic acids greater than 100  
5 nucleotides in length is difficult using automated methods, and the therapeutic cost of such molecules is prohibitive. In this invention, small nucleic acid motifs (e.g., antisense oligonucleotides, hammerhead or the hairpin ribozymes) are used for exogenous delivery.  
10 The simple structure of these molecules increases the ability of the nucleic acid to invade targeted regions of the mRNA structure. However, these nucleic acid molecules can also be expressed within cells from eukaryotic promoters (e.g., Izant and Weintraub, 1985  
15 Science 229, 345; McGarry and Lindquist, 1986 Proc. Natl. Acad. Sci. USA 83, 399; SullengerScanlon et al., 1991, Proc. Natl. Acad. Sci. USA, 88, 10591-5; Kashani-Sabet et al., 1992 Antisense Res. Dev., 2, 3-15; Dropulic et al., 1992 J. Virol., 66, 1432-41;  
20 Weerasinghe et al., 1991 J. Virol., 65, 5531-4; Ojwang et al., 1992 Proc. Natl. Acad. Sci. USA 89, 10802-6; Chen et al., 1992 Nucleic Acids Res., 20, 4581-9; Sarver et al., 1990 Science 247, 1222-1225; Thompson et al., 1995 Nucleic Acids Res. 23, 2259). Those skilled  
25 in the art realize that any nucleic acid can be expressed in eukaryotic cells from the appropriate DNA/RNA vector. The activity of such nucleic acids can be augmented by their release from the primary transcript by a ribozyme (Draper et al., PCT WO93/23569,  
30 and Sullivan et al., PCT WO94/02595, both hereby incorporated in their totality by reference herein; Ohkawa et al., 1992 Nucleic Acids Symp. Ser., 27, 15-6; Taira et al., 1991, Nucleic Acids Res., 19, 5125-30;

Ventura et al., 1993 *Nucleic Acids Res.*, 21, 3249-55; Chowrira et al., 1994 *J. Biol. Chem.* 269, 25856).

Such ribozymes are useful for the prevention of the diseases and conditions discussed above, and any other 5 diseases or conditions that are related to the levels of c-fos activity in a cell or tissue.

By "related" is meant that the inhibition of c-fos RNAs and thus reduction in the level respective protein activity will relieve to some extent the symptoms of the 10 disease or condition.

Ribozymes are added directly, or can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to target cells. The nucleic acid or nucleic acid complexes can be locally administered to 15 relevant tissues ex vivo, or in vivo through injection, infusion pump or stent, with or without their incorporation in biopolymers. In preferred embodiments, the ribozymes have binding arms which are complementary to the sequences in Tables III and IV. Examples of such 20 ribozymes are also shown in Tables III and IV. Examples of such ribozymes consist essentially of sequences defined in these Tables.

By "consists essentially of" is meant that the active ribozyme contains an enzymatic center or core 25 equivalent to those in the examples, and binding arms able to bind mRNA such that cleavage at the target site occurs. Other sequences may be present which do not interfere with such cleavage.

Thus, in a first aspect, the invention features 30 ribozymes that inhibit gene expression and/or cell proliferation. These chemically or enzymatically synthesized RNA molecules contain substrate binding domains that bind to accessible regions of their target mRNAs. The RNA molecules also contain domains that

catalyze the cleavage of RNA. The RNA molecules are preferably ribozymes of the hammerhead or hairpin motif. Upon binding, the ribozymes cleave the target mRNAs, preventing translation and protein accumulation. In the 5 absence of the expression of the target gene, cell proliferation is inhibited.

In a preferred embodiment, the enzymatic RNA molecules cleave *c-fos* mRNA and inhibit cell proliferation. Such ribozymes are useful for the 10 prevention and/or treatment of cancer. Ribozymes are added directly, or can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to smooth muscle cells. The RNA or RNA complexes can be locally administered to relevant 15 tissues through the use of a catheter, infusion pump or stent, with or without their incorporation in bio-polymers. The ribozymes, similarly delivered, also are useful for inhibiting proliferation of certain cancers associated with elevated levels of the *c-fos* oncogene, 20 particularly leukemias, neuroblastomas, and lung, colon, and breast carcinomas. Using the methods described herein, other enzymatic RNA molecules that cleave *c-fos* and thereby inhibit tumor cell proliferation may be derived and used as described above. Specific examples 25 are provided below in the Tables and figures.

In another aspect of the invention, ribozymes that cleave target molecules and inhibit *c-fos* activity are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors are preferably DNA 30 plasmids or viral vectors. Ribozyme expressing viral vectors could be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. Preferably, the recombinant vectors capable of expressing the ribozymes are delivered as described

above, and persist in target cells. Alternatively, viral vectors may be used that provide for transient expression of ribozymes. Such vectors might be repeatedly administered as necessary. Once expressed, 5 the ribozymes cleave the target mRNA. Delivery of ribozyme expressing vectors could be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or 10 by any other means that would allow for introduction into the desired target cell (for a review see Couture and Stinchcomb, 1996, *TIG.*, 12, 510).

By "patient" is meant an organism which is a donor or recipient of explanted cells or the cells themselves. 15 "Patient" also refers to an organism to which enzymatic nucleic acid molecules can be administered. Preferably, a patient is a mammal or mammalian cells. More preferably, a patient is a human or human cells.

By "vectors" is meant any nucleic acid- and/or 20 viral-based technique used to deliver a desired nucleic acid.

These ribozymes, individually, or in combination or in conjunction with other drugs, can be used to treat diseases or conditions discussed above. For example, to 25 treat a disease or condition associated with c-fos levels, the patient may be treated, or other appropriate cells may be treated, as is evident to those skilled in the art.

In a further embodiment, the described ribozymes 30 can be used in combination with other known treatments to treat conditions or diseases discussed above. For example, the described ribozymes could be used in combination with one or more known therapeutic agents to treat cancer.

In preferred embodiments, the ribozymes have binding arms which are complementary to the sequences in the tables, shown as Seq. I.D. Nos. 1-140 and 325-368. Examples of such ribozymes are shown as Seq. I.D. Nos. 5 141-324. Other sequences may be present which do not interfere with such cleavage.

In another aspect of the invention, ribozymes that cleave target molecules and inhibit cell proliferation are expressed from transcription units inserted into 10 DNA, RNA, or viral vectors. Preferably, the recombinant vectors capable of expressing the ribozymes are locally delivered as described above, and transiently persist in smooth muscle cells. Once expressed, the ribozymes 15 cleave their target mRNAs and prevent proliferation of their host cells. The recombinant vectors are preferably DNA plasmids or adenovirus vectors. However, other mammalian cell vectors that direct the expression of RNA may be used for this purpose.

Other features and advantages of the invention will 20 be apparent from the following description of the preferred embodiments thereof, and from the claims.

#### Description Of The Preferred Embodiments

The drawings will first briefly be described.

#### Drawings:

25 Figure 1 is a diagrammatic representation of the hammerhead ribozyme domain known in the art. Stem II can be ≥2 base-pair long.

Figure 2a is a diagrammatic representation of the hammerhead ribozyme domain known in the art; Figure 2b 30 is a diagrammatic representation of the hammerhead ribozyme as divided by Uhlenbeck (1987, *Nature*, 327,

596-600) into a substrate and enzyme portion; Figure 2c is a similar diagram showing the hammerhead divided by Haseloff and Gerlach (1988, *Nature*, 334, 585-591) into two portions; and Figure 2d is a similar diagram showing 5 the hammerhead divided by Jeffries and Symons (1989, *Nucl. Acids. Res.*, 17, 1371-1371) into two portions.

Figure 3 is a diagrammatic representation of the general structure of a hairpin ribozyme. Helix 2 (H2) is provided with at least 4 base pairs (i.e., n is 1, 2, 10 3 or 4) and helix 5 can be optionally provided of length 2 or more bases (preferably 3-20 bases, i.e., m is from 1-20 or more). Helix 2 and helix 5 may be covalently linked by one or more bases (i.e., r is  $\geq 1$  base). Helix 1, 4 or 5 may also be extended by 2 or more base pairs 15 (e.g., 4-20 base pairs) to stabilize the ribozyme structure, and preferably is a protein binding site. In each instance, each N and N' independently is any normal or modified base and each dash represents a potential base-pairing interaction. These nucleotides may be 20 modified at the sugar, base or phosphate. Complete base-pairing is not required in the helices, but is preferred. Helix 1 and 4 can be of any size (i.e., o and p is each independently from 0 to any number, e.g., 20) as long as some base-pairing is maintained. 25 Essential bases are shown as specific bases in the structure, but those in the art will recognize that one or more may be modified chemically (abasic, base, sugar and/or phosphate modifications) or replaced with another base without significant effect. Helix 4 can be formed 30 from two separate molecules, i.e., without a connecting loop. The connecting loop when present may be a ribonucleotide with or without modifications to its base, sugar or phosphate. "q" is  $\geq 2$  bases. The

connecting loop can also be replaced with a non-nucleotide linker molecule. H refers to bases A, U, or C. Y refers to pyrimidine bases. "—" refers to a covalent bond.

5 Figure 4 is a representation of the general structure of the hepatitis delta virus ribozyme domain known in the art.

Figure 5 is a representation of the general structure of the self-cleaving VS RNA ribozyme domain.

10 Figure 6 is a graphical representation of results of an RNA cleavage reaction catalyzed by ribozymes targeted against c-fos RNA at the indicated sites. Numbers 163-1178 are meant to indicate examples of nucleotide sites within c-fos RNA that are targeted for 15 ribozyme cleavage.

Figure 7 is a graphical representation of c-fos c-allyl ribozyme-mediated inhibition of cell proliferation. Numbers 166-1359 are meant to indicate examples of nucleotide sites within c-fos RNA that are targeted 20 for ribozyme cleavage. 6/6 and 4/10 arms are meant to indicate the number of nucleotides in each of the two binding arms of a hammerhead ribozyme targeted against site 193.

Figure 8 is a graphical representation of c-fos 25 amino ribozyme-mediated inhibition of cell proliferation.

Figure 9 shows generic structures of chemically modified hammerhead ribozymes. A) diagrammatic representation of Amino hammerhead ribozyme. B) 30 diagrammatic representation of C-allyl hammerhead ribozyme.

Target sites

Targets for useful ribozymes can be determined as disclosed in Draper et al., WO 93/23569; Sullivan et al., WO 93/23057; Thompson et al., WO 94/02595; Draper et al., WO 95/04818; McSwiggen et al., US Patent No. 5,525,468 and hereby incorporated by reference herein in totality. Rather than repeat the guidance provided in those documents here, below are provided specific examples of such methods, not limiting to those in the art. Ribozymes to such targets are designed as described in those applications and synthesized to be tested *in vitro* and *in vivo*, as also described. Such ribozymes can also be optimized and delivered as described therein.

The sequence of human *c-fos* mRNAs were screened for optimal ribozyme target sites using a computer folding algorithm. Hammerhead or hairpin ribozyme cleavage sites were identified. These sites are shown in Tables III and IV (All sequences are 5' to 3' in the tables) The nucleotide base position is noted in the Tables as that site to be cleaved by the designated type of ribozyme. The nucleotide base position is noted in the Tables as that site to be cleaved by the designated type of ribozyme.

Hammerhead or hairpin ribozymes were designed that could bind and were individually analyzed by computer folding (Jaeger et al., 1989 *Proc. Natl. Acad. Sci. USA*, 86, 7706) to assess whether the ribozyme sequences fold into the appropriate secondary structure. Those ribozymes with unfavorable intramolecular interactions between the binding arms and the catalytic core are eliminated from consideration. Varying binding arm lengths can be chosen to optimize activity. Generally,

at least 5 bases on each arm are able to bind to, or otherwise interact with, the target RNA.

Ribozymes of the hammerhead or hairpin motif were designed to anneal to various sites in the mRNA message.

5 The binding arms are complementary to the target site sequences described above. The ribozymes were chemically synthesized. The method of synthesis used follows the procedure for normal RNA synthesis as described in Usman et al., 1987 *J. Am. Chem. Soc.*, 109, 10 7845; Scaringe et al., 1990 *Nucleic Acids Res.*, 18, 5433; and Wincott et al., 1995 *Nucleic Acids Res.* 23, 2677-2684 and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end.

15 Small scale synthesis were conducted on a 394 Applied Biosystems, Inc. synthesizer using a modified 2.5  $\mu\text{mol}$  scale protocol with a 5 min coupling step for alkylsilyl protected nucleotides and 2.5 min coupling step for 2'-O-methylated nucleotides. Table II outlines the 20 amounts, and the contact times, of the reagents used in the synthesis cycle. A 6.5-fold excess (163  $\mu\text{L}$  of 0.1 M = 16.3  $\mu\text{mol}$ ) of phosphoramidite and a 24-fold excess of S-ethyl tetrazole (238  $\mu\text{L}$  of 0.25 M = 59.5  $\mu\text{mol}$ ) relative to polymer-bound 5'-hydroxyl was used in each 25 coupling cycle. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, were 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer: 30 detritylation solution was 2% TCA in methylene chloride (ABI); capping was performed with 16% N-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation solution was 16.9 mM  $\text{I}_2$ , 49 mM

pyridine, 9% water in THF (Millipore). B & J Synthesis Grade acetonitrile was used directly from the reagent bottle. S-Ethyl tetrazole solution (0.25 M in acetonitrile) was made up from the solid obtained from 5 American International Chemical, Inc.

Deprotection of the RNA was performed as follows. The polymer-bound oligoribonucleotide, trityl-off, was transferred from the synthesis column to a 4mL glass screw top vial and suspended in a solution of 10 methylamine (MA) at 65°C for 10 min. After cooling to -20°C, the supernatant was removed from the polymer support. The support was washed three times with 1.0 mL of EtOH:MeCN:H<sub>2</sub>O/3:1:1, vortexed and the supernatant was then added to the first supernatant. The combined 15 supernatants, containing the oligoribonucleotide, were dried to a white powder.

The base-deprotected oligoribonucleotide was resuspended in anhydrous TEA•HF/NMP solution (250 μL of a solution of 1.5mL N-methylpyrrolidinone, 750 μL TEA 20 and 1.0 mL TEA•3HF to provide a 1.4M HF concentration) and heated to 65°C for 1.5h. The resulting, fully deprotected, oligomer was quenched with 50 mM TEAB (9 mL) prior to anion exchange desalting.

For anion exchange desalting of the deprotected 25 oligomer, the TEAB solution was loaded onto a Qiagen 500® anion exchange cartridge (Qiagen Inc.) that was prewashed with 50 mM TEAB (10 mL). After washing the loaded cartridge with 50 mM TEAB (10 mL), the RNA was eluted with 2 M TEAB (10 mL) and dried down to a white 30 powder.

Inactive hammerhead ribozymes were synthesized by substituting a U for G5 and a U for A<sub>14</sub> (numbering from

Hertel, K. J., et al., 1992, Nucleic Acid Res., 20, 3252).

The average stepwise coupling yields were >98% (Wincott et al., 1995 *Nucleic Acids Res.* 23, 2677-5 2684).

Hairpin ribozymes are synthesized in two parts and annealed to reconstruct the active ribozyme (Chowrira and Burke, 1992 *Nucleic Acids Res.*, 20, 2835-2840).

Ribozymes are also synthesized from DNA templates using 10 bacteriophage T7 RNA polymerase (Milligan and Uhlenbeck, 1989, *Methods Enzymol.* 180, 51).

Ribozymes are modified to enhance stability and/or enhance catalytic activity by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-15 flouro, 2'-O-methyl, 2'-H, nucleotide base modifications (for a review see Usman and Cedergren, 1992 *TIBS* 17, 34; Usman et al., 1994 *Nucleic Acids Symp. Ser.* 31, 163; Burgin et al., 1996 *Biochemistry* 6, 14090). Ribozymes are purified by gel electrophoresis using general 20 methods or are purified by high pressure liquid chromatography (HPLC; See Wincott et al., *supra*) the totality of which is hereby incorporated herein by reference) and are resuspended in water.

The sequences of the ribozymes that are chemically 25 synthesized, useful in this study, are shown in Tables III-IV. Those in the art will recognize that these sequences are representative only of many more such sequences where the enzymatic portion of the ribozyme (all but the binding arms) is altered to affect 30 activity. For example, stem-loop II sequence of hammerhead ribozymes can be altered (substitution, deletion, and/or insertion) to contain any sequences provided a minimum of two base-paired stem structure can

form. Similarly, stem-loop IV sequence of hairpin ribozymes listed in Tables IV (5'-CACGUUGUG-3') can be altered (substitution, deletion, and/or insertion) to contain any sequence, provided a minimum of two base-paired stem structure can form. Preferably, no more than 200 bases are inserted at these locations. The sequences listed in Tables II and IV may be formed of ribonucleotides or other nucleotides or non-nucleotides. Such ribozymes (which have enzymatic activity) are equivalent to the ribozymes described specifically in the Tables.

#### Optimizing Ribozyme Activity

Ribozyme activity can be optimized as described by Draper et al., *supra*. The details will not be repeated here, but include altering the length of the ribozyme binding arms (stems I and III, see Figure 2c), or chemically synthesizing ribozymes with modifications (base, sugar and/or phosphate) that prevent their degradation by serum ribonucleases and/or enhance their enzymatic activity (see e.g., Eckstein et al., International Publication No. WO 92/07065; Perrault et al., 1990 *Nature* 344, 565; Pieken et al., 1991 *Science* 253, 314; Usman and Cedergren, 1992 *Trends in Biochem. Sci.* 17, 334; Usman et al., International Publication No. WO 93/15187; and Rossi et al., International Publication No. WO 91/03162; Sproat, US Patent No. 5,334,711; and Burgin et al., *supra*; all of these describe various chemical modifications that can be made to the base, phosphate and/or sugar moieties of enzymatic RNA molecules). Modifications which enhance their efficacy in cells, and removal of stem II bases to shorten RNA synthesis times and reduce chemical

requirements are desired. (All these publications are hereby incorporated by reference herein.).

By "enhanced enzymatic activity" is meant to include activity measured in cells and/or *in vivo* where 5 the activity is a reflection of both catalytic activity and ribozyme stability. In this invention, the product of these properties is increased or not significantly (less than 10 fold) decreased *in vivo* compared to an all RNA ribozyme.

10 The enzymatic nucleic acid having chemical modifications which maintain or enhance enzymatic activity is provided. Such nucleic acid is also generally more resistant to nucleases than unmodified nucleic acid. By "modified bases" in this aspect is 15 meant nucleotide bases other than adenine, guanine, cytosine and uracil at 1' position or their equivalents; such bases may be used within the catalytic core of the enzyme as well as in the substrate-binding regions. In particular, the invention features modified ribozymes 20 having a base substitution selected from pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyluracil, dihydrouracil, naphthyl, 6-methyl-uracil and aminophenyl. As noted above, substitution in the core may decrease *in vitro* activity 25 but enhances stability. Thus, in a cell and/or *in vivo* the activity may not be significantly lowered. As exemplified herein such ribozymes are useful in a cell and/or *in vivo* even if activity over all is reduced 10 fold. Such ribozymes herein are said to "maintain" the 30 enzymatic activity on all RNA ribozyme.

Sullivan, et al., *supra*, describes the general methods for delivery of enzymatic RNA molecules. Ribozymes may be administered to cells by a variety of

methods known to those familiar to the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins, biodegradable 5 nanocapsules, and bioadhesive microspheres. For some indications, ribozymes may be directly delivered *ex vivo* to cells or tissues with or without the aforementioned vehicles. Alternatively, the RNA/vehicle combination is locally delivered by direct injection or by use of a 10 catheter, infusion pump or stent. Other routes of delivery include, but are not limited to, intravascular, intramuscular, subcutaneous or joint injection, aerosol inhalation, oral (tablet or pill form), topical, systemic, ocular, intraperitoneal and/or intrathecal 15 delivery. More detailed descriptions of ribozyme delivery and administration are provided in Sullivan *et al.*, *supra* and Draper *et al.*, *supra* which have been incorporated by reference herein.

Another means of accumulating high concentrations 20 of a ribozyme(s) within cells is to incorporate the ribozyme-encoding sequences into a DNA or RNA expression vector. Transcription of the ribozyme sequences are driven from a promoter for eukaryotic RNA polymerase I (pol I), RNA polymerase II (pol II), or RNA polymerase 25 III (pol III). Transcripts from pol II or pol III promoters will be expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type will depend on the nature of the gene regulatory sequences (enhancers, silencers, etc.) present nearby. Prokaryotic RNA polymerase promoters are also used, providing that the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells (Elroy-Stein and Moss, 1990 *Proc. Natl. Acad. Sci. U S A*, 87, 6743-7; 30 Gao and Huang 1993 *Nucleic Acids Res.*, 21, 2867-72;

Lieber et al., 1993 *Methods Enzymol.*, 217, 47-66; Zhou et al., 1990 *Mol. Cell. Biol.*, 10, 4529-37). Several investigators have demonstrated that ribozymes expressed from such promoters can function in mammalian cells  
5 (e.g. Kashani-Sabet et al., 1992 *Antisense Res. Dev.*, 2, 3-15; Ojwang et al., 1992 *Proc. Natl. Acad. Sci. USA*, 89, 10802-6; Chen et al., 1992 *Nucleic Acids Res.*, 20, 4581-9; Yu et al., 1993 *Proc. Natl. Acad. Sci. USA*, 90, 6340-4; L'Huillier et al., 1992 *EMBO J.* 11, 10 4411-8; Lisziewicz et al., 1993 *Proc. Natl. Acad. Sci. USA*, 90, 8000-4; Thompson et al., 1995 *Nucleic Acids Res.* 23, 2259). The above ribozyme transcription units can be incorporated into a variety of vectors for introduction into mammalian cells, including but not  
15 restricted to, plasmid DNA vectors, viral DNA vectors (such as adenovirus or adeno-associated virus vectors), or viral RNA vectors (such as retroviral or alphavirus vectors) (for a review see Couture and Stinchcomb, 1996, *supra*).

20 In a preferred embodiment of the invention, a transcription unit expressing a ribozyme that cleaves mRNAs encoded by *c-fos* is inserted into a plasmid DNA vector or an adenovirus or adeno-associated virus DNA viral vector or a retroviral RNA vector. Viral vectors  
25 have been used to transfer genes and lead to either transient or long term gene expression (Zabner et al., 1993 *Cell* 75, 207; Carter, 1992 *Curr. Opin. Biotech.* 3, 533). The adenovirus vector is delivered as recombinant adenoviral particles. The DNA may be delivered alone or  
30 complexed with vehicles (as described for RNA above). The recombinant adenovirus or AAV particles are locally administered to the site of treatment, *e.g.*, through incubation or inhalation *in vivo* or by direct

application to cells or tissues ex vivo. Retroviral vectors have also been used to express ribozymes in mammalian cells (Ojwang et al., 1992 *supra*; Thompson et al., 1995 *supra*; Couture and Stinchcomb, 1996, *supra*).

5 In another preferred embodiment, the ribozyme is administered to the site of *c-fos* expression (e.g., tumor cells) in an appropriate liposomal vesicle.

#### Examples

10 Ability Of Ribozymes Directed Against *c-fos* RNA To Modulate gene expression and Cell Proliferation

The following examples demonstrate the selection of ribozymes that cleave *c-fos* RNA. The methods described herein represent a scheme by which ribozymes may be derived that cleave other RNA targets required for cell division. Also provided is a description of how such ribozymes may be delivered to cells. The examples demonstrate that upon delivery, the ribozymes inhibit cell proliferation in culture and modulate gene expression in vivo. Moreover, significantly reduced inhibition is observed if mutated ribozymes that are catalytically inactive are applied to the cells. Thus, inhibition requires the catalytic activity of the ribozymes.

25 Example 1: Identification of Potential Ribozyme Cleavage Sites in Human *c-fos* RNA

The sequence of human *c-fos* RNA was screened for accessible sites using a computer folding algorithm. Regions of the mRNA that did not form secondary folding structures and contained potential hammerhead and/or hairpin ribozyme cleavage sites were identified. The

sequences of these cleavage sites are shown in tables III and IV.

Example 2: Selection of Ribozyme Cleavage Sites in Human c-fos RNA

5 To test whether the sites predicted by the computer-based RNA folding algorithm corresponded to accessible sites in c-fos RNA, 14 hammerhead sites were selected for analysis. Ribozyme target sites were chosen by analyzing genomic sequences of human c-fos (GenBank  
10 Accession No. K00650 and GenBank Accession No. M16287, respectively; van Straaten et al., 1983, *Proc. Natl. Acad. Sci. USA*, 80, 3183) and prioritizing the sites on the basis of folding. Hammerhead ribozymes were designed that could bind each target (see Figure 2C) and  
15 were individually analyzed by computer folding (Christoffersen et al., 1994 *J. Mol. Struc. Theochem*, 311, 273; Jaeger et al., 1989, *Proc. Natl. Acad. Sci. USA*, 86, 7706) to assess whether the ribozyme sequences fold into the appropriate secondary structure. Those  
20 ribozymes with unfavorable intramolecular interactions between the binding arms and the catalytic core were eliminated from consideration. As noted below, varying binding arm lengths can be chosen to optimize activity. Generally, at least 5 bases on each arm are able to bind  
25 to, or otherwise interact with, the target RNA.

Example 3: Chemical Synthesis and Purification of Ribozymes for Efficient Cleavage of c-fos RNA

Ribozymes of the hammerhead or hairpin motif were designed to anneal to various sites in the RNA message.  
30 The binding arms are complementary to the target site sequences described above. The ribozymes were

chemically synthesized. The method of synthesis used followed the procedure for normal RNA synthesis as described in Usman et al., (1987 *J. Am. Chem. Soc.*, 109, 7845), Scaringe et al., (1990 *Nucleic Acids Res.*, 18, 5433) and Wincott et al., *supra*, and made use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. The average stepwise coupling yields were >98%. Inactive ribozymes were synthesized by substituting a U for G<sub>5</sub> and a U for A<sub>14</sub> (numbering from Hertel et al., 1992 *Nucleic Acids Res.*, 20, 3252). Hairpin ribozymes were synthesized in two parts and annealed to reconstruct the active ribozyme (Chowrira and Burke, 1992 *Nucleic Acids Res.*, 20, 2835-2840). Ribozymes were also synthesized from DNA templates using bacteriophage T7 RNA polymerase (Milligan and Uhlenbeck, 1989, *Methods Enzymol.* 180, 51). All ribozymes were modified to enhance stability by modification with nuclease resistant groups, for example, 2'-amino, 2'-C-allyl, 2'-flouro, 2'-O-methyl, 2'-H (for a review see Usman and Cedergren, 1992 *TIBS* 17, 34). Ribozymes were purified by gel electrophoresis using general methods or were purified by high pressure liquid chromatography (HPLC; See Wincott et al., *supra*; the totality of which is hereby incorporated herein by reference) and were resuspended in water. The sequences of the chemically synthesized ribozymes used in this study are shown below in Table III and IV.

Example 4: Ribozyme Cleavage of c-fos RNA Target

Fourteen hammerhead-type ribozymes targeted to the human c-fos RNA were designed and synthesized to test the cleavage activity *in vitro*. The target sequences

and the nucleotide location within the c-fos mRNA are given in Table III. All hammerhead ribozymes were synthesized with binding arm (Stems I and III; see Figure 2C) lengths of seven nucleotides. The relative abilities of these ribozymes to cleave human c-fos RNA is summarized in Figure 6.

Full-length or partially full-length, internally-labeled target RNA for ribozyme cleavage assay was prepared by *in vitro* transcription in the presence of [α-<sup>32</sup>P] CTP, passed over a G 50 Sephadex column by spin chromatography and used as substrate RNA without further purification. Alternately, substrates were 5'-<sup>32</sup>P-end labeled using T4 polynucleotide kinase enzyme. Assays were performed by pre-warming a 2X concentration of purified ribozyme in ribozyme cleavage buffer (50 mM Tris-HCl, pH 7.5 at 37°C, 10 mM MgCl<sub>2</sub>) and the cleavage reaction was initiated by adding the 2X ribozyme mix to an equal volume of substrate RNA (maximum of 1-5 nM) that was also pre-warmed in cleavage buffer. As an initial screen, assays were carried out for 1 hour at 37°C using a final concentration of either 40 nM or 1 μM ribozyme, i.e., ribozyme excess. The reaction was quenched by the addition of an equal volume of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol after which the sample was heated to 95°C for 2 minutes, quick chilled and loaded onto a denaturing polyacrylamide gel. Substrate RNA and the specific RNA cleavage products generated by ribozyme cleavage were visualized on an autoradiograph of the gel. The percentage of cleavage was determined by Phosphor Imager® quantitation of bands representing the intact substrate and the cleavage products.

As shown in Figure 6, all 14 hammerhead ribozymes cleaved their target RNAs in a sequence-specific manner.

Example 5: Ability of c-fos Ribozymes to Inhibit Smooth Muscle Cell Proliferation.

5       The ribozymes that cleaved c-fos RNA described above were assayed for their effect on smooth muscle cell proliferation. Human vascular smooth muscle cells were isolated and cultured as follows. Human aortic smooth muscle cells (AOSMC) were obtained from Clonetics and were grown in SmGM (Clonetics®). Cells from passage five or six were used for assays. For the cell proliferation assays, 24-well tissue culture plates were prepared by coating the wells with 0.2% gelatin and washing once with phosphate-buffered saline (PBS).  
10      AOSMC were inoculated at  $1 \times 10^4$  cells per well in 1 ml of SmGM plus 10% FBS and additives and incubated for 24 hours. The cells were subconfluent when plated at this density. The cells were serum-starved by removing the medium, washing once with PBS, and incubating 48-72  
15      hours in SmBM plus 0.5% FBS.  
20      

In several other systems, cationic lipids have been shown to enhance the bioavailability of oligonucleotides to cells in culture (Bennet, C. F., et al., 1992, Mol. Pharmacology, 41, 1023-1033). In many of the following  
25      experiments, ribozymes were complexed with cationic lipids. The cationic lipid, Lipofectamine (a 3:1 (w/w) formulation of DOSPA (2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate) and dioleoyl phosphatidylethanolamine  
30      (DOPE)), was purchased from Life Technologies, Inc. DMRIE (*N*-[1-(2,3-ditetra-decyloxy)propyl]-*N,N*-dimethyl-*N*-hydroxyethylammonium bromide) was obtained from VICAL.

DMRIE was resuspended in CHCl<sub>3</sub> and mixed at a 1:1 molar ratio with dioleoyl phosphatidylethanolamine (DOPE). The CHCl<sub>3</sub> was evaporated, the lipid was resuspended in water, vortexed for 1 minute and bath sonicated for 5 minutes. 5 Ribozyme and cationic lipid mixtures were prepared in serum-free DMEM immediately prior to addition to the cells. DMEM plus additives was warmed to room temperature (about 20-25°C), cationic lipid was added to the final desired concentration and the solution was vortexed briefly. RNA oligonucleotides were added to the final desired concentration and the solution was again vortexed briefly and incubated for 10 minutes at room temperature. In dose response experiments, the RNA/lipid complex was serially diluted 10 into DMEM following the 10 minute incubation.

Serum-starved smooth muscle cells were washed twice with PBS, and the RNA/lipid complex was added. The plates were incubated for 4 hours at 37°C. The medium was then removed and DMEM containing 10% FBS, additives 20 and 10 µM bromodeoxyuridine (BrdU) was added. In some wells, FBS was omitted to determine the baseline of unstimulated proliferation. The plates were incubated at 37°C for 20-24 hours, fixed with 0.3% H<sub>2</sub>O<sub>2</sub> in 100% methanol, and stained for BrdU incorporation by standard 25 methods. In this procedure, cells that have proliferated and incorporated BrdU stain brown; non-proliferating cells are counter-stained a light purple. Both BrdU positive and BrdU negative cells were counted under the microscope. 300-600 total cells per well were 30 counted. In the following experiments, the percentage of the total cells that have incorporated BrdU (% cell proliferation) is presented. Errors represent the range of duplicate wells. Percent inhibition then is calcu-

lated from the % cell proliferation values as follows:  
% inhibition =  $100 - \frac{100((\text{Ribozyme} - 0\% \text{ serum}) / (\text{Control} - 0\% \text{ serum}))}{100}$ .

Fifteen stabilized hammerhead ribozymes with C-allyl modification (see Figure 9B) were delivered at a concentration of 0.3  $\mu$ M, complexed with DMRIE/DOPE such that the cationic lipid charges and the anionic RNA charges were at 1:1 molar ratio. The results, shown in Figure 7, demonstrate the efficacy of ribozymes directed against different sites within c-fos RNA. The control, inactive ribozymes that cannot cleave c-fos RNA due to alterations in their catalytic core sequence fail to inhibit smooth muscle cell proliferation. Thus, inhibition of cell proliferation by these hammerhead sequences is due to their ability to cleave c-fos RNA, and not because of any non-ribozyme activity.

Example 6: Ribozymes Inhibit Proliferation of Smooth Muscle Cells in a Dose-Dependent Fashion.

If the inhibition of proliferation observed in Example 5 is caused by the ribozymes, the level of inhibition should be proportional to the dose of RNA added. Human aortic smooth muscle cells were assayed for proliferation in the presence of differing doses of an amino (see Figure 9A) site 268 hammerhead ribozymes. The result shown in Figure 8, indicates that two hammerhead ribozymes that cleave c-fos RNA at sites 268 inhibit SMC proliferation in a dose-dependent fashion. Ribozymes were delivered with the cationic lipid, Lipofectamine at a 1:1 charge ratio. In this experiment, 10% FBS (no ribozyme) gave  $92 \pm 1\%$  proliferation; 0% FBS gave  $6 \pm 1\%$  proliferation. The control is an inactive ribozyme and shows no inhibition over the dose range tested. The control ribozyme

contains the same catalytic core sequences as the active ribozymes but differs in its catalytic core sequence. Thus, ribozyme inhibition of smooth muscle cell proliferation requires sequence-specific binding and 5 cleavage by the hammerhead ribozymes targeted against c-fos RNA.

Example 7: Modulation of Stromelysin Gene Expression in Rabbit Knee by c-fos Ribozyme.

In order to extend the ribozyme efficacy in cell culture, applicant has chosen to use rabbit knee as a reasonable animal model to study ribozyme-mediated inhibition of rabbit stromelysin protein expression. Applicant selected a amino hammerhead (HH) ribozyme (268 Amino Ribozyme), targeted to site 268 within human c-fos 15 RNA, for animal studies. This has enabled applicant to compare the efficacy of the same ribozyme in human as well as in rabbit systems.

Male New Zealand White Rabbits (3-4 Kg) were anaesthetized with ketamine-HCl/xylazine and injected 20 intra-articularly (I.T.) in both knees with 100  $\mu$ g ribozyme (268 Amino Ribozyme) in 0.5 ml phosphate buffered saline (PBS) or PBS alone (Controls). The IL-1 (human recombinant IL-1 $\alpha$ , 25 ng) was administered I.T., 24 hours following the ribozyme administration. Each 25 rabbit received IL-1 in one knee and PBS alone in the other. The synovium was harvested 6 hours post IL-1 infusion, snap frozed in liquid nitrogen, and stored at -80°C. Total RNA is extracted with TRIzol reagent (GIBCO BRL, Gaithersburg, MD), and was analyzed by 30 Northern-blot analysis and/or RNase-protection assay. Briefly, 0.5  $\mu$ g cellular RNA was separated on 1.0 % agarose/formaldehyde gel and transferred to Zeta-Probe GT nylon membrane (Bio-Rad, Hercules, CA) by capillary

transfer for ~16 hours. The blots were baked for two hours and then pre-hybridized for 2 hours at 65°C in 10 ml Church hybridization buffer (7 % SDS, 500 mM phosphate, 1 mM EDTA, 1% Bovine Serum Albumin). The 5 blots were hybridized at 65°C for ~16 hours with 10<sup>6</sup> cpm/ml of full length <sup>32</sup>P-labeled complementary RNA (cRNA) probes to rabbit stromelysin mRNA (cRNA added to the pre-hybridization buffer along with 100  $\mu$ l 10mg/ml salmon sperm DNA). The blot was rinsed once with 5% 10 SDS, 25 mM phosphate, 1 mM EDTA and 0.5% BSA for 10 min at room temperature. This was followed by two washes (10 min each wash) with the same buffer at 65°C, which was then followed by two washes (10 min each wash) at 65°C with 1% SDS, 25 mM phosphate and 1 mM EDTA. The 15 blot was autoradiographed. The blot was reprobed with a 100 nt cRNA probe to 18S rRNA as described above. Following autoradiography, the stromelysin expression was quantified on a scanning densitometer, which is followed by normalization of the data to the 18S rRNA 20 band intensities.

As shown in Table V, catalytically active 268 Amino Ribozyme mediates a decrease in the expression of stromelysin RNA in rabbit knees. The inhibition appears to be sequence-specific and ranges from 40-47%.

25 Optimizing Ribozyme Activity

Sullivan, et al., *supra*, describes the general methods for delivery of enzymatic RNA molecules. The data presented in Examples above indicate that different cationic lipids can deliver active ribozymes 30 to smooth muscle cells. Experiments similar to those performed in above-mentioned Examples are used to determine which lipids give optimal delivery of

ribozymes to specific cells. Other such delivery methods are known in the art and can be utilized in this invention.

The proliferation of smooth muscle cells can also 5 be inhibited by the direct addition of chemically stabilized ribozymes. Presumably, uptake is mediated by passive diffusion of the anionic nucleic acid across the cell membrane. In this case, efficacy could be greatly enhanced by directly coupling a ligand to the ribozyme. 10 The ribozymes are then delivered to the cells by receptor-mediated uptake. Using such conjugated adducts, cellular uptake can be increased by several orders of magnitude without having to alter the phosphodiester linkages necessary for ribozyme cleavage 15 activity.

Alternatively, ribozymes may be administered to cells by a variety of methods known to those familiar to the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation 20 into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres. The RNA/vehicle combination is locally delivered by direct injection or by use of a catheter, infusion pump or stent. Alternative routes of delivery 25 include, but are not limited to, intramuscular injection, aerosol inhalation, oral (tablet or pill form), topical, systemic, ocular, intraperitoneal and/or intrathecal delivery. More detailed descriptions of ribozyme delivery and administration are provided in 30 Sullivan, et al., *supra* and Draper, et al., *supra* which have been incorporated by reference herein.

Chemical modifications, ribozyme sequences and ribozyme motifs described in this invention are meant to be non-limiting examples, and those skilled in the art

will recognize that other modifications (base, sugar and phosphate modifications) to enhance nuclease stability of a ribozyme can be readily generated using standard techniques and are hence within the scope of this  
5 invention.

Use of Ribozymes Targeting c-fos

Overexpression of the *c-fos* oncogene has been reported in a number of cancers (see above). Thus, inhibition of *c-fos* expression (for example using  
10 ribozymes) can reduce cell proliferation of a number of cancers, *in vitro* and *in vivo* and can reduce their proliferative potential. A cascade of MMP and serine proteinase expression is implicated in the acquisition of an invasive phenotype as well as in angiogenesis in  
15 tumors (MacDougall & Matrisian, 1995, *Cancer & Metastasis Reviews* 14, 351;Ritchlin & Winchester, 1989,  
*Springer Semin Immunopathol.*, 11, 219).

Ribozymes, with their catalytic activity and increased site specificity (see above), are likely to  
20 represent a potent and safe therapeutic molecule for the treatment of cancer. In the present invention, ribozymes are shown to inhibit smooth muscle cell proliferation and stromelysin gene expression. From those practiced in the art, it is clear from the  
25 examples described, that the same ribozymes may be delivered in a similar fashion to cancer cells to block their proliferation.

Chronic wound healing: Metalloproteinase expression is undetectable in normal epidermis, but is stimulated  
30 upon wounding. Although protease expression is required for tissue remodelling in normal wound healing, it is likely than excessive proteolytic activity contributes to the pathology of chronic ulcers. Collagenase

expression in basal keratinocytes at the advancing edge of wounds is correlated with degree of ulceration (Saarialho-Kere et al., 1992, *J. Clin. Invest.* 90:1952) and has been implicated in the pathophysiology of 5 chronic blistering disease, recessive dystrophic epidermolysis bullosa and skin cancer (Lin et al., 1995, *FASEB J.* 9, 1371-77). Stromelysin 1 and 2 are both expressed by keratinocytes in a variety of chronic ulcers. Since *c-fos* regulates the expression of all of 10 these MMPs, ribozymes targeting *c-fos* could potentially lead to enhanced re-epithelialization of ulcers.

Arthritis: Matrix metalloproteinases have frequently been implicated in the degradation of cartilage associated with both rheumatoid and 15 osteoarthritis (Hembry et al., 1995, *Ann. Rheum. Dis.*, 54, 25-32; Okada et al., 1992, *Lab. Invest.* 66, 680). Since multiple MMPs appear to contribute to the destructive phenotype, the ability to inhibit the entire 20 MMP family would be desirable. In addition, *c-fos* has been shown to be required for MHC class I expression (Kushtai et al., 1988, *Oncogene* 2,119). Thus, inhibition of *c-fos* expression by ribozymes would likely reduce both the cartilage catabolism associated with MMP expression and also the underlying immune response 25 triggering rheumatoid arthritis. *C-fos* ribozymes therefore show considerable promise as therapeutics for arthritis.

Diagnostic uses

Ribozymes of this invention may be used as 30 diagnostic tools to examine genetic drift and mutations within diseased cells or to detect the presence of *c-fos* RNA in a cell. The close relationship between ribozyme

activity and the structure of the target RNA allows the detection of mutations in any region of the molecule which alters the base-pairing and three-dimensional structure of the target RNA. By using multiple 5 ribozymes described in this invention, one may map nucleotide changes which are important to RNA structure and function *in vitro*, as well as in cells and tissues. Cleavage of target RNAs with ribozymes may be used to inhibit gene expression and define the role 10 (essentially) of specified gene products in the progression of disease. In this manner, other genetic targets may be defined as important mediators of the disease. These experiments will lead to better 15 treatment of the disease progression by affording the possibility of combinational therapies (e.g., multiple ribozymes targeted to different genes, ribozymes coupled with known small molecule inhibitors, or intermittent treatment with combinations of ribozymes and/or other chemical or biological molecules). Other *in vitro* uses 20 of ribozymes of this invention are well known in the art, and include detection of the presence of mRNAs associated with *c-fos*-related condition. Such RNA is detected by determining the presence of a cleavage product after treatment with a ribozyme using standard 25 methodology.

In a specific example, ribozymes which can cleave only wild-type or mutant forms of the target RNA are used for the assay. The first ribozyme is used to identify wild-type RNA present in the sample and the 30 second ribozyme will be used to identify mutant RNA in the sample. As reaction controls, synthetic substrates of both wild-type and mutant RNA will be cleaved by both ribozymes to demonstrate the relative ribozyme efficiencies in the reactions and the absence . of

cleavage of the "non-targeted" RNA species. The cleavage products from the synthetic substrates will also serve to generate size markers for the analysis of wild-type and mutant RNAs in the sample population.

5 Thus each analysis will require two ribozymes, two substrates and one unknown sample which will be combined into six reactions. The presence of cleavage products will be determined using an RNase protection assay so that full-length and cleavage fragments of each RNA can

10 be analyzed in one lane of a polyacrylamide gel. It is not absolutely required to quantify the results to gain insight into the expression of mutant RNAs and putative risk of the desired phenotypic changes in target cells.

15 The expression of mRNA whose protein product is implicated in the development of the phenotype (i.e., c-fos) is adequate to establish risk. If probes of comparable specific activity are used for both transcripts, then a qualitative comparison of RNA levels will be adequate and will decrease the cost of the

20 initial diagnosis. Higher mutant form to wild-type ratios will be correlated with higher risk whether RNA levels are compared qualitatively or quantitatively.

Other embodiments are within the following claims.

Table I

## TABLE I:

Characteristics of naturally occurring ribozymesGroup I Introns

- Size: ~150 to >1000 nucleotides.
- 5 • Requires a U in the target sequence immediately 5' of the cleavage site.
- Binds 4-6 nucleotides at the 5'-side of the cleavage site.
- Reaction mechanism: attack by the 3'-OH of guanosine to generate cleavage products with 3'-OH and 5'-guanosine.
- 10 • Additional protein cofactors required in some cases to help folding and maintenance of the active structure [1].
- Over 300 known members of this class. Found as an intervening sequence in *Tetrahymena thermophila* rRNA, fungal mitochondria, chloroplasts, phage T4, blue-green algae, and others.
- 15 • Major structural features largely established through phylogenetic comparisons, mutagenesis, and biochemical studies [2,3].
- Complete kinetic framework established for one ribozyme [4,5,6,7].
- 20 • Studies of ribozyme folding and substrate docking underway [8,9,10].
- Chemical modification investigation of important residues well established [11,12].
- 25 • The small (4-6 nt) binding site may make this ribozyme too non-specific for targeted RNA cleavage, however, the *Tetrahymena* group I intron has been used to repair a "defective"  $\beta$ -galactosidase message by the ligation of new  $\beta$ -galactosidase sequences onto the defective message [13].
- 30
- 35 RNase P RNA (M1 RNA)
  - Size: ~290 to 400 nucleotides.
  - RNA portion of a ubiquitous ribonucleoprotein enzyme.
  - Cleaves tRNA precursors to form mature tRNA [14]. Reaction mechanism: possible attack by  $M^{2+}$ -OH to

Table I

generate cleavage products with 3'-OH and 5'-phosphate.

- RNAse P is found throughout the prokaryotes and eukaryotes. The RNA subunit has been sequenced from bacteria, yeast, rodents, and primates.
- Recruitment of endogenous RNAse P for therapeutic applications is possible through hybridization of an External Guide Sequence (EGS) to the target RNA [15,16]
- Important phosphate and 2' OH contacts recently identified [17,18]

Group II Introns

- Size: >1000 nucleotides.
- Trans cleavage of target RNAs recently demonstrated [19,20].
- Sequence requirements not fully determined.
- Reaction mechanism: 2'-OH of an internal adenosine generates cleavage products with 3'-OH and a "lariat" RNA containing a 3'-5' and a 2'-5' branch point.
- Only natural ribozyme with demonstrated participation in DNA cleavage [21,22] in addition to RNA cleavage and ligation.
- Major structural features largely established through phylogenetic comparisons [23].
- Important 2' OH contacts beginning to be identified [24]
- Kinetic framework under development [25]

Neurospora VS RNA

- Size: ~144 nucleotides.
- Trans cleavage of hairpin target RNAs recently demonstrated [26].
- Sequence requirements not fully determined.
- Reaction mechanism: attack by 2'-OH 5' to the scissile bond to generate cleavage products with 2',3'-cyclic phosphate and 5'-OH ends.
- Binding sites and structural requirements not fully determined.
- Only 1 known member of this class. Found in Neurospora VS RNA.

Table I

Hammerhead Ribozyme

(see text for references)

- Size: ~13 to 40 nucleotides.
- Requires the target sequence UH immediately 5' of the cleavage site.
- Binds a variable number nucleotides on both sides of the cleavage site.
- Reaction mechanism: attack by 2'-OH 5' to the scissile bond to generate cleavage products with 2',3'-cyclic phosphate and 5'-OH ends.
- 14 known members of this class. Found in a number of plant pathogens (virusoids) that use RNA as the infectious agent.
- Essential structural features largely defined, including 2 crystal structures [24]
- Minimal ligation activity demonstrated (for engineering through *in vitro* selection) [25]
- Complete kinetic framework established for two or more ribozymes [26].
- Chemical modification investigation of important residues well established [27].

Hairpin Ribozyme

- Size: ~50 nucleotides.
- Requires the target sequence GUC immediately 3' of the cleavage site.
- Binds 4-6 nucleotides at the 5'-side of the cleavage site and a variable number to the 3'-side of the cleavage site.
- Reaction mechanism: attack by 2'-OH 5' to the scissile bond to generate cleavage products with 2',3'-cyclic phosphate and 5'-OH ends.
- 3 known members of this class. Found in three plant pathogen (satellite RNAs of the tobacco ringspot virus, arabis mosaic virus and chicory yellow mottle virus) which uses RNA as the infectious agent.
- Essential structural features largely defined [27,28,29,30]
- Ligation activity (in addition to cleavage activity) make ribozyme amenable to engineering through *in vitro* selection [31]
- Complete kinetic framework established for one ribozyme [32].

Table I

- Chemical modification investigation of important residues begun [33,34].

Hepatitis Delta Virus (HDV) Ribozyme

- Size: ~60 nucleotides.
- Trans cleavage of target RNAs demonstrated [35].
- Binding sites and structural requirements not fully determined, although no sequences 5' of cleavage site are required. Folded ribozyme contains a pseudoknot structure [36].
- Reaction mechanism: attack by 2'-OH 5' to the scissile bond to generate cleavage products with 2',3'-cyclic phosphate and 5'-OH ends.
- Only 2 known members of this class. Found in human HDV.
- Circular form of HDV is active and shows increased nuclease stability [37].

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**Table II: 2.5  $\mu$ mol RNA Synthesis Cycle**

Reagent	Equivalents	Amount	Wait Time*
Phosphoramidites	6.5	163 $\mu$ L	2.5
S-Ethyl Tetrazole	23.8	238 $\mu$ L	2.5
Acetic Anhydride	100	233 $\mu$ L	5 sec
N-Methyl Imidazole	186	233 $\mu$ L	5 sec
TCA	83.2	1.73 mL	21 sec
Iodine	8.0	1.18 mL	45 sec
Acetonitrile	NA	6.67 mL	NA

\* Wait time does not include contact time during delivery.

Table III

TABLE III: Human C-fos Hammerhead Ribozyme and Target Sequences

nt Position	Target site	SEQ. ID.NOS	Ribozyme Sequence	SEQ. ID.NOS
9	AACCGCAUC UGCAGCGA	1	UCGCUGCA CUGAUGA X GAA AUGCGGUU	141
80	ACCGUGCUC CUACCCAG	2	CUGGGUAG CUGAUGA X GAA AGCACGGU	142
83	GUCCUCCUA CCCAGCUC	3	GAGCUGGG CUGAUGA X GAA AGGACCCAC	143
91	ACCCAGCUC UGCCUUCAC	4	GUGAAGCA CUGAUGA X GAA AGCUGGGU	144
96	GCUCUGCUU CAACAGCGC	5	GCGCUGUG CUGAUGA X GAA AGCAGAGC	145
97	CUCUGCUUC ACAGCGCC	6	GGCGCUGU CUGAUGA X GAA AGCAGAG	146
113	CCACCCUGUC UCCCCCCC	7	GGGGCGGA CUGAUGA X GAA ACAGGUGG	147
115	ACCUGUCUC CGCCCCUC	8	GAGGGGCG CUGAUGA X GAA AGACAGGU	148
123	CCGCCCCUC GGCCCCUC	9	GAGGGGCC CUGAUGA X GAA AGGGCGG	149
131	CGGCCCCUC GCCGGCU	10	AGCCGGGC CUGAUGA X GAA AGGGGGCG	150
140	GCCCCGGCUU UGCCUAAC	11	GUUAGGC CUGAUGA X GAA AGCCCCGGC	151
141	CCCGGCUUU GCCUAACC	12	GGUUAGGC CUGAUGA X GAA AGGCGGG	152
146	CUUUGCCUA ACCGCCAC	13	GUGGCGGU CUGAUGA X GAA AGGCAAAG	153
163	GAUGAUGUU CUCGGGU	14	AGCCCCAG CUGAUGA X GAA ACAUCAUC	154
164	AUGAUGUUC UCGGGCUU	15	AAGCCCGA CUGAUGA X GAA ACAUCAU	155
172	CUCGGGCUU CAACCGAG	16	CUGCGUUG CUGAUGA X GAA AGCCCGAG	156
173	UCGGGCUUC AACGCAGA	17	UCUGCGUU CUGAUGA X GAA AGGCGCGA	157
184	CGCAGACUA CGAGGCGU	18	ACGCCUUG CUGAUGA X GAA AGUCUGCG	158
196	GGCGUCAUC CUCCCGCU	19	AGCGGGAG CUGAUGA X GAA AUGACGCC	159
199	GUCAUCCUC CCCUGCGA	20	UGCAGCGG CUGAUGA X GAA AGGAUGAC	160
217	CAGCGCGUC CCCGGCG	21	CGGCCGGG CUGAUGA X GAA ACGCGCUG	161
231	CCGGGGAUU GCCUCUCU	22	AGAGAGGC CUGAUGA X GAA AUCCCCGG	162
236	GAUAGCCUC UCUUACUA	23	UAGUAAGA CUGAUGA X GAA AGGCUAUC	163
238	UAGCCUCUC AUACUACC	24	GGUAGUAA CUGAUGA X GAA AGAGGCCUA	164
240	GCCUCUCUU AUACUACC	25	GUGGUAGU CUGAUGA X GAA AGAGAGGC	165
241	CCUCUCUUA AUACCUAC	26	AGUGGGAG CUGAUGA X GAA AAGAGAGG	166
244	CUCUUAUCU CCACUCAC	27	GUGAGUGG CUGAUGA X GAA AGUAAGAG	167
250	CUACCAUCU ACCCGCAG	28	CUGCGGGU CUGAUGA X GAA AGUGGUAG	168
262	CGCAGACUCU CUUCUCCA	29	UGGAGAAAG CUGAUGA X GAA AGUCUGCG	169
265	AGACUCCUU CUCCAGCA	30	UGCUGGAG CUGAUGA X GAA AGGAGUCU	170
266	GACUCCUUC UCCAGCAU	31	AUGCUGGA CUGAUGA X GAA AAGGAGUC	171
268	CUCCUUCUC CAGCAUGG	32	CCAUUCUG CUGAUGA X GAA AGAAGGAG	172
280	CAUGGGCUC GCCUGUCA	33	UGACAGGC CUGAUGA X GAA AGCCCAUG	173
287	UCGCCUGUC AACCGCAGA	34	UGCCGCUU CUGAUGA X GAA ACAGGCGA	174
301	GCAGGACUUC UCGCACCG	35	CCGGCAG CUGAUGA X GAA AGUCCUGC	175
302	CGAGGACUUC UGCACCGA	36	UCCGUGCA CUGAUGA X GAA AGUCCUG	176
320	CUGGCCGUC UCCAGUGC	37	GCACUGGA CUGAUGA X GAA ACGGCCAG	177
322	GGCCGCUUC CAGUGCCA	38	UGGCACUG CUGAUGA X GAA AGACGGCC	178
334	UGCCAACUUU CAUUCCCA	39	UGGGAAGU CUGAUGA X GAA AGUUGGCA	179
335	GCCAACUUC AUUCCCCAC	40	UGGGGAU CUGAUGA X GAA AGUUGGC	180
338	AACUUCAUU CCCACGGU	41	ACCGUGGG CUGAUGA X GAA AUGAAGUU	181
339	ACUUCAUUC CCACGGUC	42	GACCGUGG CUGAUGA X GAA AAUGAAGU	182
347	CCCACGGUC ACUGCCAU	43	AUGGCAGU CUGAUGA X GAA ACCGUGGG	183
356	ACUGCCAUUC UCGACCCAG	44	CUGGGCAG CUGAUGA X GAA AUGGCAGU	184
358	UGCCAUCUC GACCAGUC	45	GACUGGUC CUGAUGA X GAA AGAUGGCA	185
366	CGACCAUCU CGGACCCUG	46	CAGGUCCG CUGAUGA X GAA ACUGGUCCG	186
398	CCCGCCCCUC GUCUCCUC	47	GAGGAGAC CUGAUGA X GAA AGGGCGGG	187
401	GCCCUCGUC UCCUCUGU	48	ACAGAGGA CUGAUGA X GAA ACGAGGGC	188
403	CCUCGUCUC CUCUGUGG	49	CCACAGAG CUGAUGA X GAA AGACGGAG	189
406	CGUCUCCUC UGUGGGCC	50	GGGCACCA CUGAUGA X GAA AGGAGACG	190
418	GGCCCCCAUC GCAGACCA	51	UGGUCCUG CUGAUGA X GAA AUGGGGCC	191
435	GAGCCCCUC ACCCUUC	52	GAAAGGGU CUGAUGA X GAA AGGGCCUC	192
441	CUCACCCUU UCGGAGUC	53	GACUCCGA CUGAUGA X GAA AGGGUGAG	193
442	UCACCCUUU CGGAGUCC	54	GGACUCGG CUGAUGA X GAA AAGGGUGA	194
443	CACCCUUUC GGAGUCC	55	GGGACUCC CUGAUGA X GAA AAAGGGUG	195
449	UUCGGAGUC CCCGCC	56	GGGGCGGG CUGAUGA X GAA ACUCGAA	196
460	CGCCCCCUC CGCUGGGG	57	CCCCACGG CUGAUGA X GAA AGGGGGCG	197
471	CUGGGCUU ACUCCAGG	58	CCUGGAGU CUGAUGA X GAA AGCCCCAG	198
472	UGGGGCUUA CUCCAGGG	59	CCCUGGAG CUGAUGA X GAA AAGCCCCA	199
475	GGCUUACUC CAGGGCUG	60	CAGCCUG CUGAUGA X GAA AGUAAGCC	200
488	GCUGGCGUU GUGAAGAC	61	GUCUUCAC CUGAUGA X GAA ACGCCAGC	201

Table III

nt Position	Target site	SEQ. ID.NOS	Ribozyme Sequence	SEQ. ID.NOS
524	CAGAGCAUU GGCAGGAG	62	CUCCUGCC CUGAUGA X GAA AUGCUCUG	202
550	GGAACAGUU AUCCUCAG	63	CUGGAGAU CUGAUGA X GAA ACUGUUC	203
551	GAACAGUUA UCUCAGA	64	UCUGGAGA CUGAUGA X GAA AACUGUUC	204
553	ACAGUUAUC UCCAGAAG	65	CUUCUGGA CUGAUGA X GAA AAUACUGU	205
555	AGUUUACUC CAGAAGAA	66	UUCUUCUG CUGAUGA GAA AGAUAACU	206
581	AGGAGAAUC CGAAGGGA	67	UCCCCUUCG CUGAUGA X GAA AUUCUCCU	207
597	AAAGGAAUA AGAUGGCC	68	AGCCAUCAU CUGAUGA X GAA AUUCUUUU	208
645	UGACUGUA CAUCCAA	69	UUGGAGUG CUGAUGA X GAA AUCAGUCA	209
650	GAUACACUC CAAGCGGA	70	UCCCCUUCG CUGAUGA X GAA AGUGUAUC	210
671	GACCAACU GAAGAUGA	71	UCAUCUUC CUGAUGA X GAA AGUUGGUC	211
685	UGAGAAUGC UGCCUUGC	72	GCAAAAGCA CUGAUGA X GAA ACUUCOCA	212
690	AGUCUGCUU UGCAGACC	73	GGUCUGCA CUGAUGA X GAA ACCAGACU	213
691	GUCUGCUU GCAGACCG	74	CGGUCCUG CUGAUGA X GAA AAGCAGAC	214
704	ACCGAGAUU GCCAACCU	75	AGGUUJGGC CUGAUGA X GAA AUCUCGGU	215
734	GAAAACUA GAGUUCAU	76	AUGAACUC CUGAUGA X GAA AGUUUUUC	216
739	ACUAGAGUU CAUCCUGG	77	CCAGGAUG CUGAUGA X GAA ACUCUAGU	217
740	CUAGAGUUC AUCCUGGC	78	GCCAGGAG CUGAUGA X GAA AACUCUAG	218
743	GAGUUCAU CUGGCAGC	79	GCUCGCG CUGAUGA X GAA AUGAACUC	219
753	UGGCAGCUC ACCGACCU	80	AGGUUCGG CUGAUGA X GAA AGCUGCCA	220
773	UGCAAGAUU CCUGAUGA	81	UCAUCAUG CUGAUGA X GAA AUCUUGCA	221
790	CCUGGGCUU CCCAGAG	82	CUUCUGGG CUGAUGA X GAA AGCCCAGG	222
791	CUGGGCUUC CCAGAAGA	83	UCUUCUGG CUGAUGA X GAA AAGCCAG	223
805	AGAGAUGUC UGUGGCUU	84	AAGCCACA CUGAUGA X GAA ACAUCU	224
813	CUGUGGCUU CCCUUGAU	85	AUCAAGG CUGAUGA X GAA AGCCACAG	225
814	UGUGGCUUC CCUUGAU	86	GAUCAAGG CUGAUGA X GAA AAGCCACA	226
818	GCUUCCCCUU GAUCUGAC	87	GUCAGAUC CUGAUGA X GAA AGGGAAUC	227
822	CCCUUGAUC UGACUGGG	88	CCCAUCUA CUGAUGA X GAA AUCAAGGG	228
845	CCAGAGGUU GCCACCCC	89	GGGGUGGC CUGAUGA X GAA ACCUCUGG	229
859	CCCGGAGUC UGAGGGAG	90	CCUUCUCA CUGAUGA X GAA ACUCCGGG	230
871	GGAGGCCUU CAUCCUGC	91	GCAGGGUG CUGAUGA X GAA AGGCCUCC	231
872	GAGGCCUUC ACCUGCC	92	GGCAGGGC CUGAUGA X GAA AAGGCCUC	232
882	CCUGGCCUC UCCUCAAU	93	AUUGAGGA CUGAUGA X GAA AGGCAGGG	233
884	CUGCCUCUC CUCAUAGA	94	UCAUUGAG CUGAUGA X GAA AGAGGAG	234
887	CCUCUCCUC AAUGACCC	95	GGGUCAUU CUGAUGA X GAA AGGAGGG	235
910	CAAGCCCCU AGUGGAAC	96	GUUCCACU CUGAUGA X GAA AGGGCUUG	236
923	GAACCUGUC AAGAGCAU	97	AUGGCUUU CUGAUGA X GAA ACAGGUUC	237
932	AAGAGCAUC AGCAGCAU	98	AUGGUGCU CUGAUGA X GAA AUGCUCUU	238
961	CGAGCCCCU UGAUGACU	99	AGUCAUCU CUGAUGA X GAA AGGGCUCG	239
962	GAGCCCCUU GAUGACU	100	AAGUCAUC CUGAUGA X GAA AAGGGCUC	240
970	UGAUGACUU CCUGUUC	101	GGAACAGG CUGAUGA X GAA AGUCAUCA	241
971	GAUGACUUC CUGUUCCC	102	GGGAACAG CUGAUGA X GAA AAGUCAUC	242
976	CUUCCUGUU CCCAGCAU	103	AUGGUGGG CUGAUGA X GAA ACAGGAAG	243
977	UCCUGUUU CCAGCAUC	104	GAUCCUGG CUGAUGA X GAA AACAGGAA	244
985	CCCAAGCAUC AUCCAGGC	105	GGCUCCAGG CUGAUGA X GAA AUGCUGGG	245
988	AGCAUCAUC CAGGCCCA	106	UGGGCCUG CUGAUGA X GAA AUGAUGCU	246
1003	CAGUGGCUC UGAGACAG	107	CUGUCUCA CUGAUGA X GAA AGCCACUG	247
1018	AGCCCCGCU CGUGCCAG	108	CUGGCACG CUGAUGA X GAA AGCGGGCU	248
1037	AUGGACCUA UCUGGGUC	109	GACCCAGA CUGAUGA X GAA AGGUCCAU	249
1039	GGACCUUAUC UGGGUCCU	110	AGGACCCA CUGAUGA X GAA AUAGGUCC	250
1045	AUCUGGGUC CUUCUAG	111	CAUAGAAG CUGAUGA X GAA ACCCAGAU	251
1048	UGGGGUCCUU CUAGCG	112	CUGCAUAG CUGAUGA X GAA AGGACCCA	252
1049	GGGUCCUUC UAUGCAGC	113	GCUGCAUA CUGAUGA X GAA AAGGACCC	253
1051	GUCCUUCUA CGAGCAG	114	CUGCUGCA CUGAUGA X GAA AGAAGGAC	254
1071	GGGAGCCUC UGCACAGU	115	ACUGUGCA CUGAUGA X GAA AGGCUCCC	255
1084	CAGUGGCUC CCUGGGGA	116	UCCCCAGG CUGAUGA X GAA AGCCACUG	256
1131	UGUGCACUC CGGUGGUC	117	GACCACCG CUGAUGA X GAA AGUGCACA	257
1139	CCGGGGUC ACCUGUAC	118	GUACAGGU CUGAUGA X GAA ACCACCGG	258
1146	UCACCUGUA CUCCCAGC	119	GCUGGGAG CUGAUGA X GAA ACAGGUGA	259
1149	CCUGUACUC CCAGCUGC	120	GCACCCUGG CUGAUGA X GAA AGUACAGG	260
1164	GCACUGCUU ACACGUU	121	-AGACGUGU CUGAUGA X GAA AGCAGUGC	261
1165	CAUCGUUA CAGGUUCU	122	AAGRCGUC CUGAUGA X GAA AAGCAGUG	262
1171	UUACACGUU UUCCUUCG	123	CGGAAGAA CUGAUGA X GAA ACGUUAA	263
1173	ACACGUUUU CCUUCGUC	124	GACCAAGG CUGAUGA X GAA AGACGUUG	264
1174	CACGUUCU CUCUGUCU	125	AGACGAAG CUGAUGA X GAA AAGACGGU	265
1177	GUCUUCUU CGUCUUC	126	UGAAAGAC CUGAUGA X GAA AGGAAGAC	266
1178	UCUUCUUC GUCUUCAC	127	GUGAAGAC CUGAUGA X GAA AAGGAAGA	267
1181	UCCUUCGUC UUCACCUA	128	UAGGUGAA CUGAUGA X GAA ACGAAGGA	268

Table III

nt Position	Target site	SEQ. ID.NOS	Ribozyme Sequence	SEQ. ID.NOS
1183	CUUCGCUUU CACCUACC	129	GGUAGGUG CUGAUGA X GAA AGACGAAG	269
1184	UUCGUCUUC ACCUACCC	130	GGGUAGGU CUGAUGA X GAA AAGACGAA	270
1189	CUUCACCUA CCCCCGAGG	131	CCUCGGGG CUGAUGA X GAA AGGUGAAG	271
1204	GGCUGACUC CUUCCCCA	132	UGGGGAAG CUGAUGA X GAA AGUCAGCC	272
1207	UGACUCUUU CCCCAGCU	133	ACCUGGGG CUGAUGA X GAA AGGAGUCA	273
1208	GACUCUUC CCCAGCUG	134	CAGCUGGG CUGAUGA X GAA AAGGAGUC	274
1257	AUGAGCCUU CUCUCUGAC	135	GUCAAGGG CUGAUGA X GAA AGGCUCAU	275
1258	UGAGCCUUC CUCUGACU	136	AGUCAGAG CUGAUGA X GAA AAGGCUCA	276
1261	GCCUUCUCU UGACUCGC	137	GGGAGUCA CUGAUGA X GAA AGGAAGGC	277
1267	CUCUGACUC GCUCAGCU	138	AGCUGAGC CUGAUGA X GAA AGUCAGAG	278
1271	GACUCGGCUC AGCUCACC	139	GGUGAGCU CUGAUGA X GAA AGCGAGUC	279
1276	GCUCAGCUC ACCCACGC	140	GCGUGGGU CUGAUGA X GAA AGCUGAGC	280

Where "X" represents stem II region of a HH ribozyme (Hertel et al., 1992 *Nucleic Acids Res.* 20 3252). The length of stem II may be ≥2 base-pairs.

Table IV

**TABLE IV: Human C-fos Hairpin Ribozyme and Target Sequence**

nt. Position	Ribozyme Sequence	SEQ. ID. NOS.	Target Sequence	SEQ. ID. NOS.
46	CGCCGC AGAA GCGG ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	281	CGGCCG GCC GCGGGCG	325
87	AAGCAG AGAA GGGU ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	282	ACCCA GCU CUGCUU	326
92	CUGUGA AGAA GAGC ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	283	GCUCU GCU UCACAG	327
110	GGCGGA AGAA GGUG ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	284	CACCU GUC UCCGCC	328
116	CCGAGG AGAA GAGA ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	285	UCUCC GGC CCUCGG	329
124	GCGAGG AGAA GAGG ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	286	CCUCG GCC CCUCGC	330
136	AGGCAA AGAA GGCG ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	287	GCCCG GCU UUGCUCU	331
179	CUCUUA AGAA GCGU ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	288	ACGCA GAC UACGAG	332
201	UGCUCG AGAA GGAG ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	289	CUCCC GCU GCAGCA	333
221	AUCCCC AGAA GGGG ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	290	CCCCG GCC GGGGAU	334
257	GAAGGA AGAA GCGG ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	291	CCGCA GAC UCCUUC	335
308	GGCCAG AGAA GUGC ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	292	GCACG GAC CUGGCC	336
317	ACUGGA AGAA GCCA ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	293	UGGCC GUC UCCAGU	338
363	GGUCCG AGAA GGUC ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	294	GACCA GUC CGGACC	338
368	CUGCAG AGAA GGAC ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	295	GUCCG GAC CUGCAG	339
388	AGGGCG AGAA GCAC ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	296	GUGCA GCC CGCCCU	340
392	GACGAG AGAA GGCU ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	297	AGCCC GCC CUCGUC	341
421	GCUCUG AGAA GCGA ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	298	UCGCA GAC CAGAGC	342
452	GGAGGG AGAA GGGA ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	299	UCCCC GCC CCCUCC	343
461	AGCCCC AGAA GAGG ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	300	CCUCC GCU GGGCU	344
637	GUAUCA AGAA GCUU ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	301	GAGCU GAC UGAUAC	345
662	UAGUUG AGAA GUCU ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	302	AGACA GAC CAACUA	346
686	CUGCAA AGAA GACU ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	303	AGUCU GCU UUGCAG	347
694	AUCUCG AGAA GCAA ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	304	UUGCA GAC CGAGAU	348
712	UCCUUC AGAA GGUU ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	305	AACCU GCU GAAGGA	349
749	UCGGUG AGAA GCGA ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	306	UGGCA GCU CACCGA	350
756	AGGCAG AGAA GUGA ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	307	UCACC GAC CUGCCU	351
761	CUUGCA AGAA GGUC ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	308	GACCU GCC UGCAAG	352
776	CAGGUC AGAA GGGG ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	309	UCCCU GAU GACCUUG	353
823	CCCCCA AGAA GAUC ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	310	GAUCU GAC UGGGGG	354
877	AGGAGA AGAA GGGU ACCAGAGAAACACACGUUGGGUACAUUACCUUGGUA	311	ACCCU GCC UCUCU	355

Table IV

nt. Position	Ribozyme Sequence	SEQ. ID. NOS.	Target Sequence	SEQ. ID. NOS.
312	GCUGGG AGAA GGAA ACCAGAGAAAACACGUUUGGGGUACAUUACCUGGUA	312	UUCCU GUU CCCAGC	356
1010	GGAGCG AGAA GU CU ACCAGAGAAAACACGUUUGGGGUACAUUACCUGGUA	313	AGACA GCC CGCUCC	357
1014	GCACGG AGAA GGC U ACCAGAGAAAACACGUUUGGGGUACAUUACCUGGUA	314	AGCCC GCU CCGUGC	358
1058	CUCCA AGAA GCUG ACCAGAGAAAACACGUUUGGGGUACAUUACCUGGUA	315	CAGCA GAC UGGGAG	359
1152	CAGUGG AGAA GGG A ACCAGAGAAAACACGUUUGGGGUACAUUACCUGGUA	316	UCCCA GCU GCACUG	360
1160	CGUGUA AGAA GUGC ACCAGAGAAAACACGUUUGGGGUACAUUACCUGGUA	317	GCACU GCU UACACG	361
1199	GAAGGA AGAA GCC U ACCAGAGAAAACACGUUUGGGGUACAUUACCUGGUA	318	AGGCU GAC UCCUUC	362
1212	CUGCAC AGAA GGG G ACCAGAGAAAACACGUUUGGGGUACAUUACCUGGUA	319	CCCCA GCU GUGCAG	363
1220	GUGGCC AGAA GCAC ACCAGAGAAAACACGUUUGGGGUACAUUACCUGGUA	320	GUGCA GCU GCCCAC	364
1223	GCGGUG AGAA GCUG ACCAGAGAAAACACGUUUGGGGUACAUUACCUGGUA	321	CAGCU GCC CACCGC	365
1262	GAGCGA AGAA GAGG ACCAGAGAAAACACGUUUGGGGUACAUUACCUGGUA	322	CCUCU GAC UCGCUC	366
1272	UGGGUG AGAA GAGC ACCAGAGAAAACACGUUUGGGGUACAUUACCUGGUA	323	GCUCA GCU CACCCA	367
1285	AGGGCC AGAA GCG U ACCAGAGAAAACACGUUUGGGGUACAUUACCUGGUA	324	ACGCU GCU GGCCCU	368

Table V: C-fos Ribozyme-Mediated Inhibition of  
Stromelysin Gene Expression In Vivo

Ribozymes	Percent Inhibition
268 Amino Ribozyme (Exp. 1)	47±10%
268 Amino Ribozyme (Exp. 2)	40±6%

Claims

1. An enzymatic nucleic acid molecule which specifically cleaves RNA derived from a *c-fos* gene, wherein said enzymatic nucleic acid molecule is in a hairpin motif.  
5
2. An enzymatic nucleic acid molecule which specifically cleaves RNA derived from a *c-fos* gene, wherein said nucleic acid molecule is in a hammerhead motif, wherein the binding arms of said  
10 nucleic acid molecule comprises sequences complementary to any of sequences defined as **SEQ. ID. NOS. 1-140.**
3. The enzymatic nucleic acid molecule of claim 2, wherein said nucleic acid molecule comprises a stem  
15 II region of length greater than or equal to 2 base pairs.
4. The enzymatic nucleic acid molecule of claim 1, wherein said nucleic hairpin motif consists essentially of any sequence selected from the  
20 sequences defined as **SEQ. ID NOS. 281-324.**
5. The enzymatic nucleic acid molecule of claim 1, wherein the binding arms of said nucleic acid molecule comprises sequences complementary to any of sequences defined as **SEQ. ID. NOS. 325-368.**
- 25 6. An enzymatic nucleic acid molecule which specifically cleaves RNA derived from a *c-fos* gene, wherein said nucleic acid molecule is in a hepatitis delta virus, VS nucleic acid, group I

intron, Group II intron, or RNase P nucleic acid motif.

7. The enzymatic nucleic acid molecule of any of claims 1, 2, or 6, wherein said nucleic acid

5 comprises between 12 and 100 bases complementary to said RNA.

8. The enzymatic nucleic acid molecule of any of claims 1, 2, or 6, wherein said nucleic acid

comprises between 14 and 24 bases complementary to 10 said mRNA.

9. The enzymatic nucleic acid molecule of claim 2, wherein said hammerhead motif consists essentially

of any sequence selected from the sequences defined as **SEQ. ID NOS. 141-280**.

15 10. A mammalian cell including an enzymatic nucleic acid molecule of any one of any of claims 1, 2, or 6.

11. The cell of claim 10, wherein said cell is a human cell.

20 12. An expression vector comprising nucleic acid sequence encoding at least one of the enzymatic nucleic acid molecule of any of claims 1, 2, or 6, in a manner which allows expression of that enzymatic nucleic acid molecule.

25 13. A mammalian cell including an expression vector of claim 12.

14. The cell of claim 13, wherein said cell is a human cell.
15. A method for treatment of cancer comprising the step of administering to a patient the enzymatic nucleic acid molecule of any of claims 1, 2, or 6.  
5
16. A method for treatment of a cancer comprising the step of administering to a patient the expression vector of claim 12.
17. A method for treatment of cancer comprising the steps of: a) isolating cells from a patient; b) administering to said cells the enzymatic nucleic acid molecule of any of claims 1, 2, 6 or 12; and c) introducing said cells back into said patient.  
10
18. A pharmaceutical composition comprising the enzymatic nucleic acid molecule of any of claims 1, 2, 6 or 12.  
15
19. A method of treatment of a patient having a condition associated with the level of *c-fos*, wherein said patient is administered the enzymatic nucleic acid molecule of any of claims 1, 2, 6 or 12.  
20
20. A method of treatment of a patient having a condition associated with the level of *c-fos*, comprising contacting cells of said patient with the nucleic acid molecule of any of claims 1, 2, 6, or 12, and further comprising the use of one or more drug therapies.  
25

21. The enzymatic nucleic acid molecule of claim 2, wherein said nucleic acid molecule comprises at least five ribose residues, and wherein said nucleic acid comprises phosphorothioate linkages at at least three of the 5' terminal nucleotides, and wherein said nucleic acid comprises a 2'-C-allyl modification at position No. 4 of said nucleic acid, and wherein said nucleic acid comprises at least ten 2'-O-methyl modifications, and wherein said nucleic acid comprises a 3'- end modification.  
5
22. The enzymatic nucleic acid of claim 21, wherein said nucleic acid comprises a 3'-3' linked inverted ribose moiety at said 3' end.
23. The enzymatic nucleic acid molecule of claim 2, wherein said nucleic acid molecule comprises at least five ribose residues, and wherein said nucleic acid molecule comprises phosphorothioate linkages at at least three of the 5' terminal nucleotides, and wherein said nucleic acid comprises a 2'-amino modification at position No. 4 and/or at position No. 7 of said nucleic acid molecule, wherein said nucleic acid molecule comprises at least ten 2'-O-methyl modifications, and wherein said nucleic acid comprises a 3'- end modification.  
15  
20  
25
24. The enzymatic nucleic acid molecule of claim 2, wherein said nucleic acid molecule comprises at least five ribose residues, and wherein said nucleic acid molecule comprises phosphorothioate linkages at at least three of the 5' terminal nucleotides, and wherein said nucleic acid molecule  
30

comprises an abasic substitution at position No. 4  
and/or at position No. 7 of said nucleic acid  
molecule, wherein said nucleic acid comprises at  
least ten 2'-O-methyl modifications, and wherein  
5       said nucleic acid molecule comprises a 3'-end  
modification.

25. The enzymatic nucleic acid molecule of claim 2,  
wherein said nucleic acid molecule comprises of at  
least five ribose residues, and wherein said  
10      nucleic acid comprises phosphorothioate linkages at  
at least three of the 5' terminal nucleotides, and  
wherein said nucleic acid molecule comprises a 6-  
methyl uridine substitution at position No. 4  
and/or at position No. 7 of said nucleic acid  
15      molecule, wherein said nucleic acid molecule  
comprises at least ten 2'-O-methyl modifications,  
and wherein said nucleic acid molecule comprises a  
3' end modification.

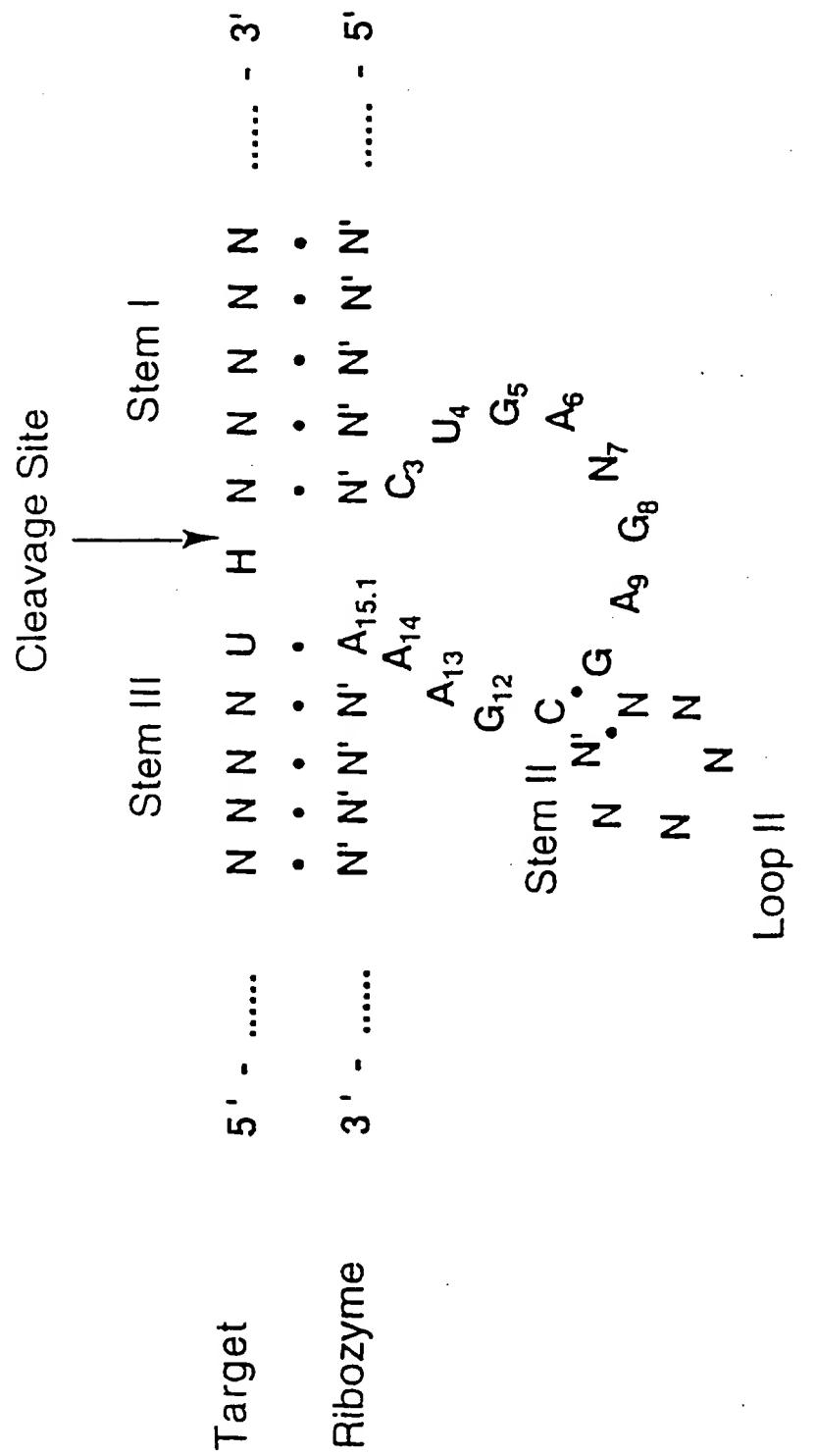
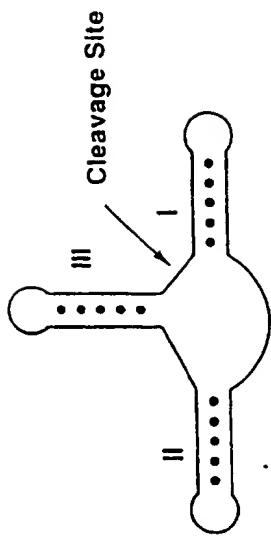
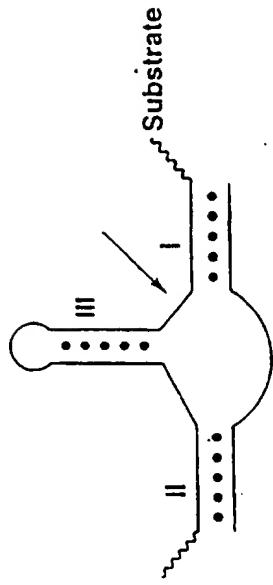


Fig. 1

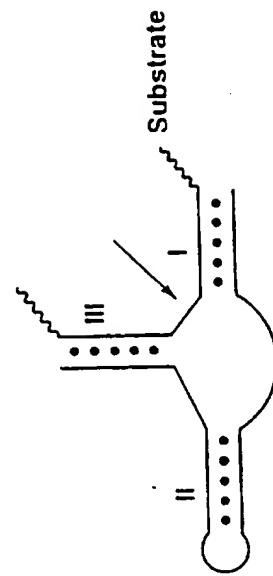
*Fig. 2A*



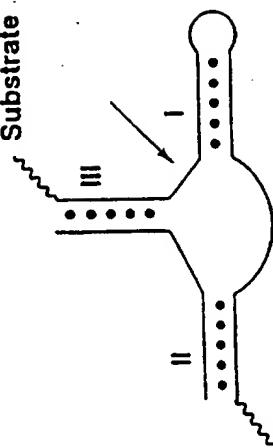
*Fig. 2B*



*Fig. 2C*



*Fig. 2D*



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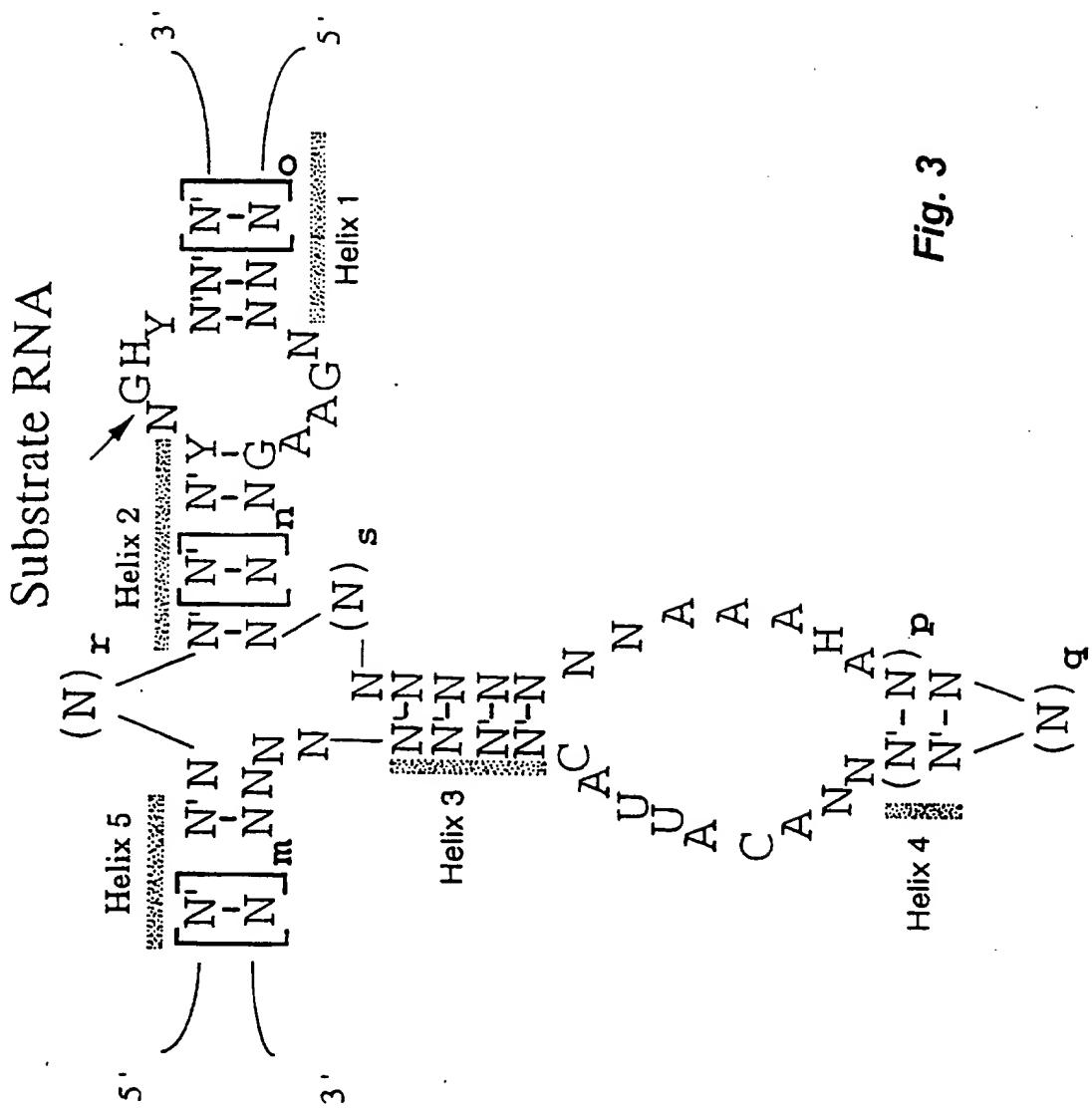
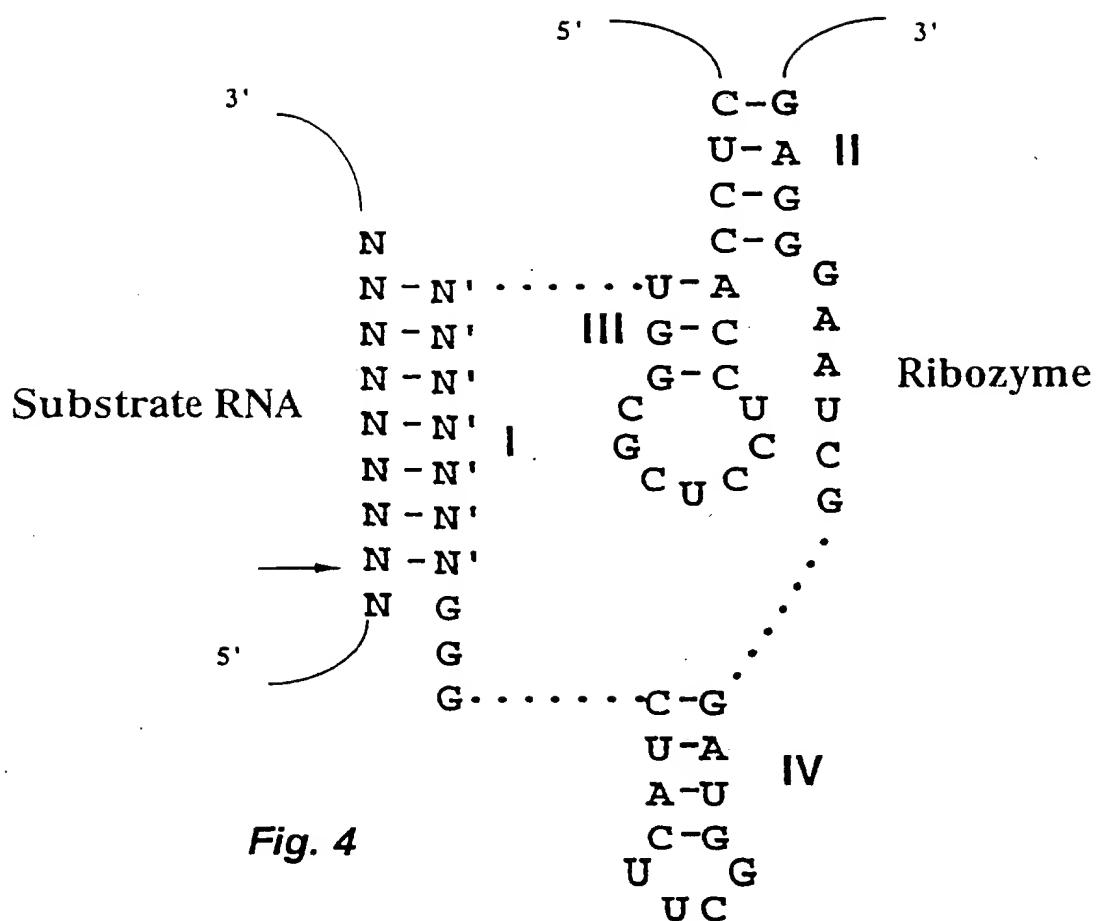


Fig. 3

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*Fig. 4*

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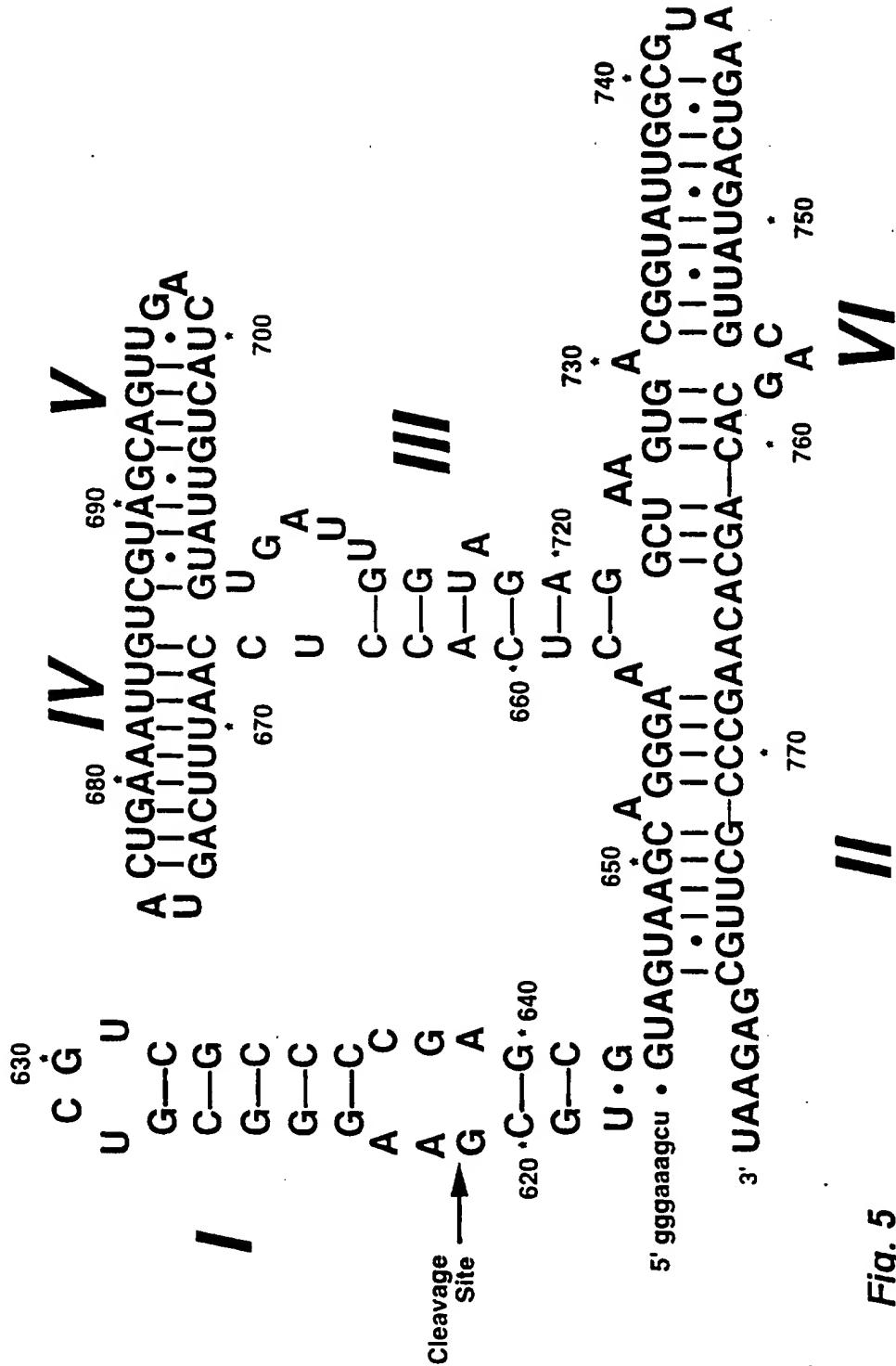


Fig. 5

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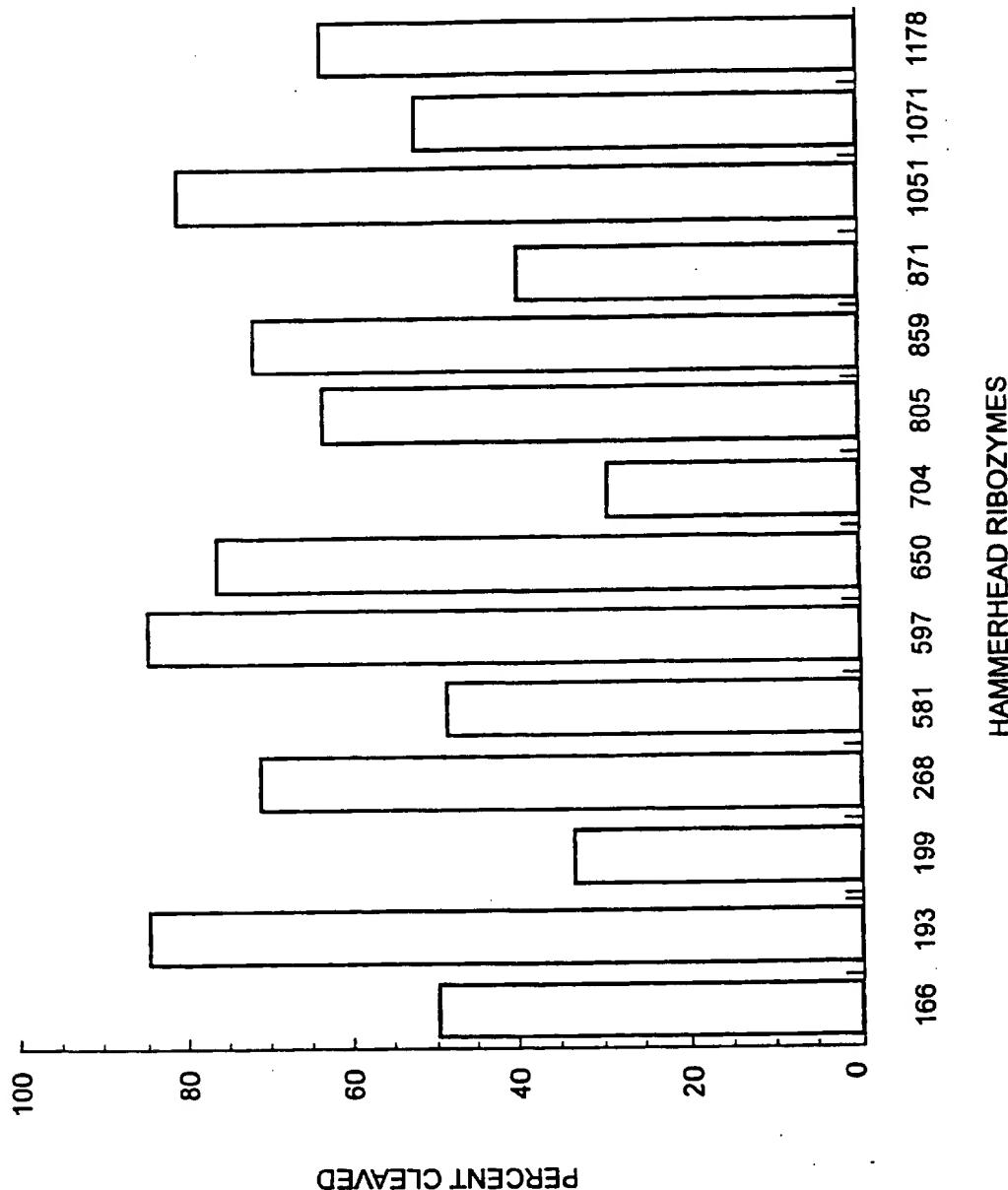


Fig. 6

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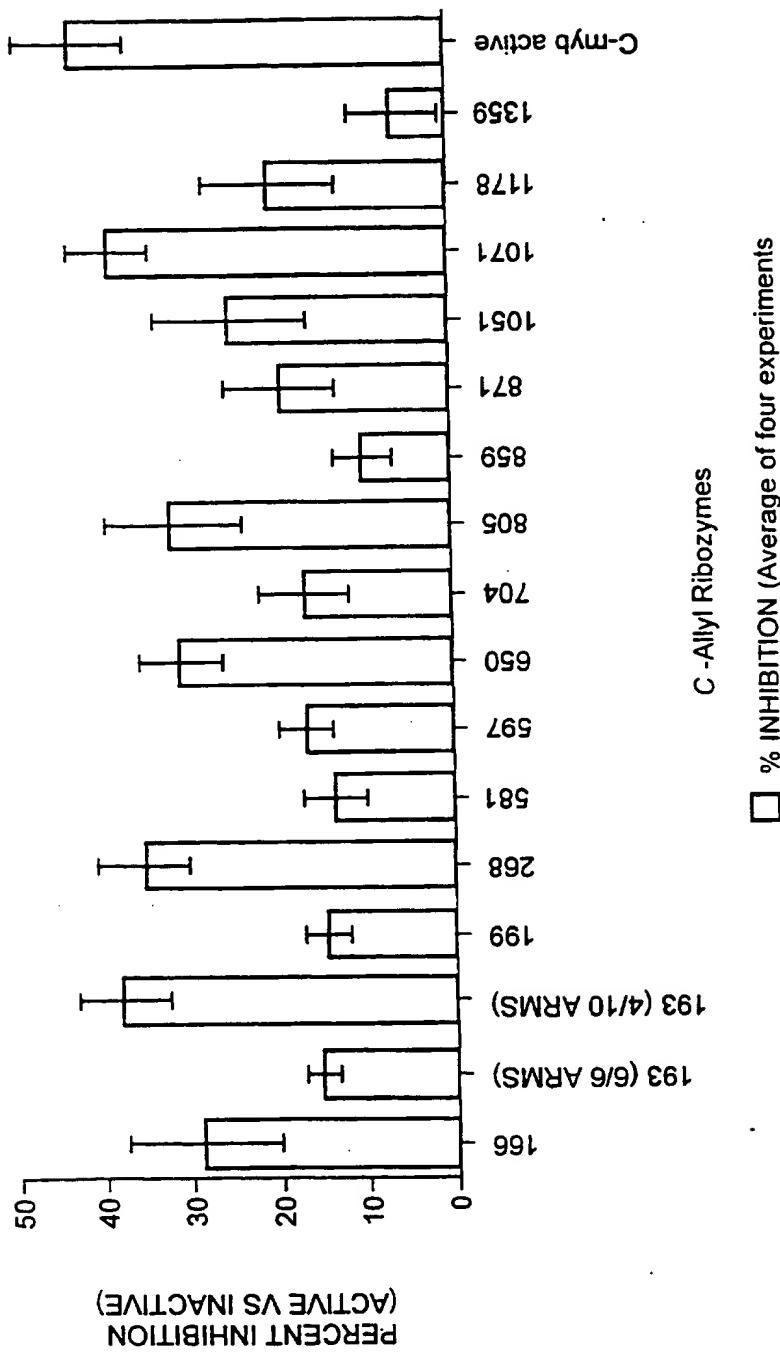


Fig. 7

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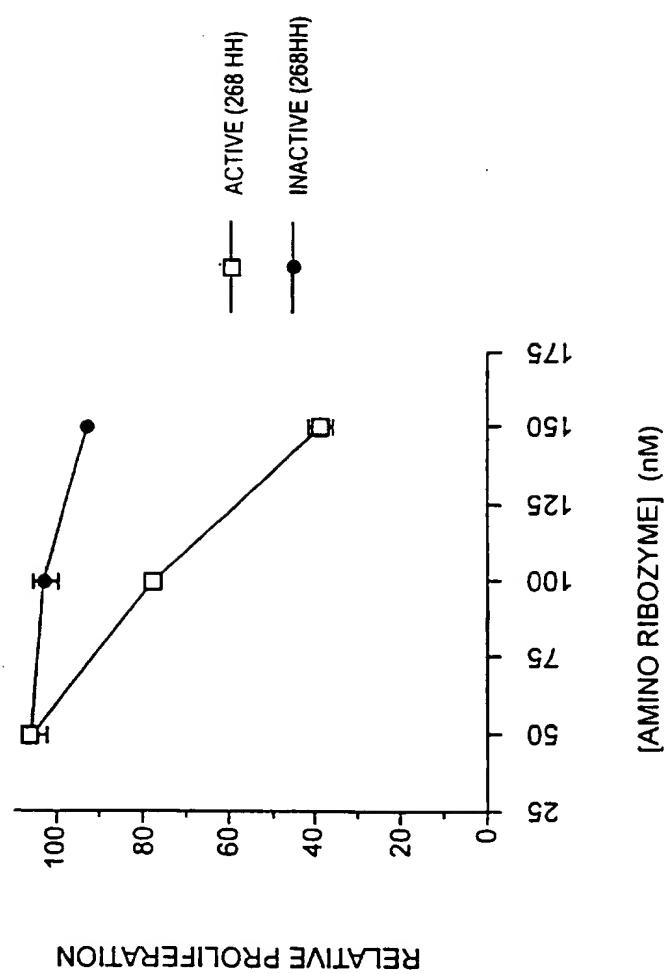
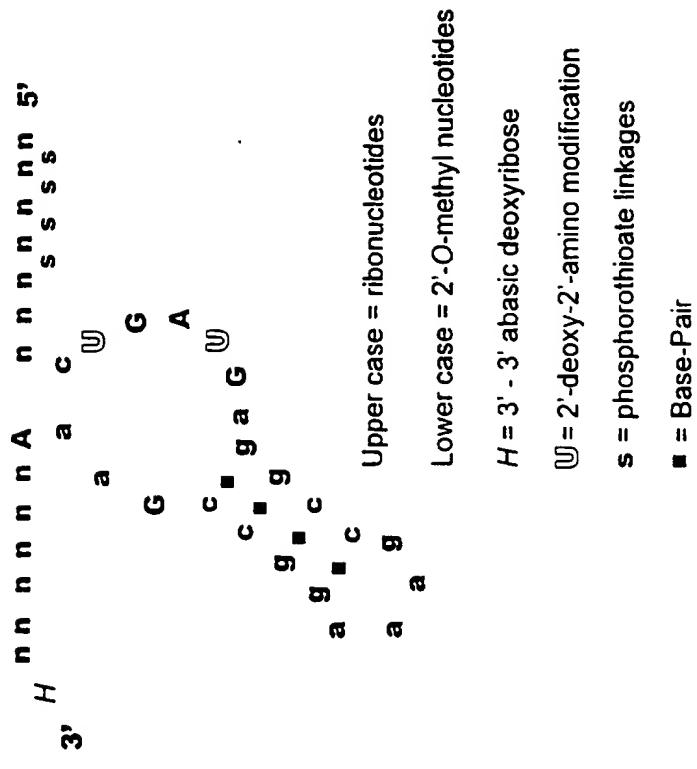


Fig. 8

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*Fig. 9A*

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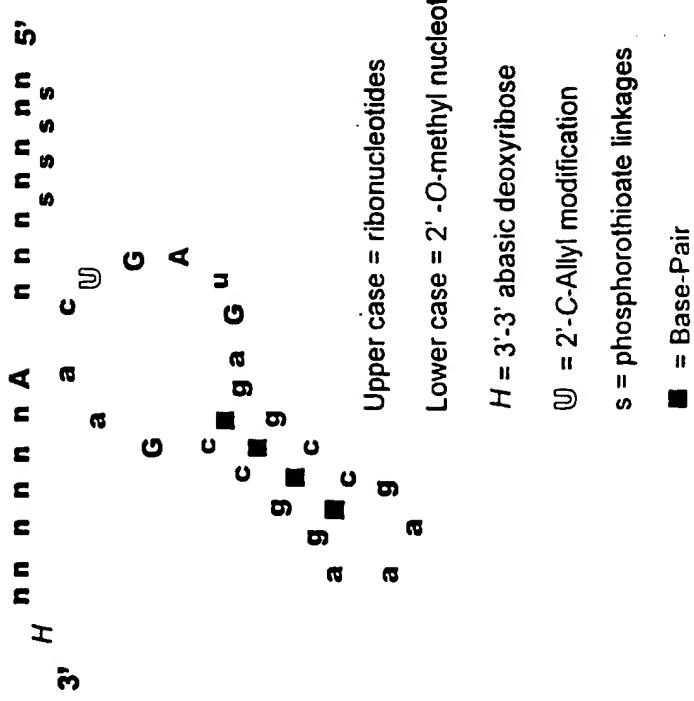


Fig. 9B

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 98/01017

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/10 C12N9/22 C12N5/10 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCANLON K J ET AL: "RIBOZYME - MEDIATED CLEAVAGE OF C - FOS mRNA REDUCES GENE EXPRESSION OF DNA SYNTHESIS ENZYMES AND METALLOTHIONEIN." PROC NATL ACAD SCI U S A 88 (23). 1991. 10591-10595. CODEN: PNASAG ISSN: 0027-8424, XP002068843 cited in the application see the whole document ---	1-25
X	WO 96 08558 A (HOPE CITY ;SCANLON KEVIN J (US)) 21 March 1996 cited in the application see page 10 - page 21 ---	1-25 -/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

## \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "Z" document member of the same patent family

Date of the actual completion of the international search

22 June 1998

Date of mailing of the international search report

25.09.98

Name and mailing address of the ISA

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Authorized officer

Chakravarty, A

## INTERNATIONAL SEARCH REPORT

International Application No PCT/US 98/01017
---

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 22689 A (UNIV COLUMBIA ;PYLE ANNA M (US); MICHELS WILLIAM J (US)) 1 August 1996 see page 29 see claim 17 ---	6-8, 10-20
X	WO 91 15580 A (RES DEV FOUNDATION) 17 October 1991 see page 7, line 11 - line 24 see page 10, line 31 - page 11, line 4 see page 11, line 33 - page 12, line 2 see examples 5-8 see claims 3,6 ---	1-25
	FUNATO T ET AL: "THE UTILITY OF AN ANTI-FOS RIBOZYME IN REVERSING CISPLATIN RESISTANCE IN HUMAN CARCINOMAS." WEBER, G. (ED.). ADVANCES IN ENZYME REGULATION, VOL. 32; THIRTY-SECOND SYMPOSIUM ON REGULATION OF ENZYME ACTIVITY AND SYNTHESIS IN NORMAL AND NEOPLASTIC TISSUES, INDIANAPOLIS, INDIANA, USA, SEPTEMBER 30-OCTOBER 1, 1991. XIV+376P. PERGAMON PRESS: OXFORD, XP002068844 ---	
P,X	WO 97 10334 A (IMMUSOL INC) 20 March 1997 see page 5, line 22 - page 8, line 13 ---	1-25
P,X	WO 97 18312 A (VIMRX HOLDINGS LTD ;LUDWIG JANOS (DE); SPROAT BRIAN (DE)) 22 May 1997 see claims 1,9,26,32 -----	1-25

**INTERNATIONAL SEARCH REPORT**

International application No.

PCT/US 98/01017

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.  Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
  
2.  Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
  
3.  Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

SUBJECT 1) CLAIMS 1,4,5,7,8,10-20 (ALL PARTIALLY)  
SUBJECT 2-88) CLAIMS 1,4,5,7,8,10-20 (ALL PARTIALLY)  
SUBJECT 89-368) CLAIMS 2,3,7-25 (ALL PARTIALLY)  
SUBJECT 369-373) CLAIMS 6-8,10-20 (ALL PARTIALLY)

1.  As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2.  As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3.  As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4.  No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1,4,5,7,8, 10-20 (ALL PARTIALLY)

**Remark on Protest** The additional search fees were accompanied by the applicant's protest. No protest accompanied the payment of additional search fees.

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

Int.	lational Application No
PCT/US 98/01017	

Patent document cited in search report		Publication date		Patent family member(s)		Publication date
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